

Influenza virus-like particles (VLPs) comprising hemagglutinin produced within a plant

FIELD OF INVENTION

5 [001] The present invention relates to the production of virus-like particles. More specifically, the present invention is directed to the production of virus-like particles comprising influenza antigens.

[002] BACKGROUND OF THE INVENTION

10 [003] Influenza is the leading cause of death in humans due to a respiratory virus. Common symptoms include fever, sore throat, shortness of breath, and muscle soreness, among others. During flu season, influenza viruses infect 10-20% of the population worldwide, leading to 250-500,000 deaths annually

15 [004] Influenza viruses are enveloped virus that bud from the plasma membrane of infected mammalian cells. They are classified into types A, B, or C, based on the nucleoproteins and matrix protein antigens present. Influenza type A viruses may be further divided into subtypes according to the combination of hemagglutinin (HA) and neuraminidase (NA) surface glycoproteins presented. HA governs the ability of the virus to bind to and penetrate the host cell. NA removes terminal sialic acid residues from glycan chains on host cell and viral surface proteins, which prevents viral aggregation and facilitates virus mobility. Currently, 16 HA (H1-H16) and 9
20 NA (N1-N9) subtypes are recognized. Each type A influenza virus presents one type of HA and one type of NA glycoprotein. Generally, each subtype exhibits species specificity; for example, all HA and NA subtypes are known to infect birds, while only subtypes H1, H2, H3, H5, H7, H9, H10, N1, N2, N3 and N7 have been shown to infect humans (Horimoto 2006; Suzuki 2005). Influenza viruses comprising H5, H7 and H9 are considered the most highly pathogenic forms of influenza A viruses, and
25 are most likely to cause future pandemics.

[005] Influenza pandemics are usually caused by highly transmittable and virulent influenza viruses, and can lead to elevated levels of illness and death globally. The emergence of new influenza A subtypes resulted in 4 major pandemics in the 20th
30 century. The Spanish flu, caused by an H1N1 virus, in 1918-1919 led to the deaths of

over 50 million people worldwide between 1917 and 1920. Presently, the risk of the emergence of a new subtype, or of the transmission to humans of a subtype endemic in animals, is always present. Of particular concern is a highly virulent form of avian influenza (also called "bird flu"), outbreaks of which have been reported in several countries around the world. In many cases, this bird flu can result in mortality rates approaching 100% within 48 hours. The spread of the avian influenza virus (H5N1), first identified in Hong Kong in 1997, to other Asian countries and Europe has been postulated to be linked to the migratory patterns of wild birds.

[006] 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 for the coming "flu-season". The prediction is coordinated by the World Health Organization. Generally, the number of vaccine doses produced each year is not sufficient to vaccinate the world's population. For example, Canada and the United-States obtain enough vaccines doses to immunize about one third of their population, while only 17% of the population of the European Union can be vaccinated. It is evident that current worldwide production of influenza vaccine would be insufficient in the face of a worldwide flu pandemic. Even if the necessary annual production 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.

[007] The viral stocks for use in vaccines are produced in fertilized eggs. The virus particles are harvested, and for an inactivated viral vaccine, disrupted by detergent to inactivate. Live attenuated vaccines are made of influenza viruses that were adapted for growth at low temperature which means that at normal body temperature, the vaccine is attenuated. Such a vaccine is licensed in USA for use in individuals from 5 to 49 years of age. Inactivated whole virus vaccines are rendered harmless by inactivation with chemical agents and they have been produced in embryonic eggs or mammalian cell culture. All these types of vaccine show some specific advantages and disadvantages. One advantage of vaccines derived from whole viruses is the type of immunity induced by such vaccines. In general, split vaccines induce a strong antibody response while vaccines made of whole viruses induce both an antibody

(humoral) and cellular response. Even though a functional antibody response is a criterion for licensure that correlates with protection induced by a vaccine, there is increasing evidence that a T-cell response is also important in influenza immunity – this may also provide better protection in the elderly.

5 [008] In order to induce a cellular immune response, vaccines made of whole viruses were developed. Due to the high pathogenicity of the influenza strain (e.g. H5N1), these vaccines are produced in BL3+ facility. For highly pathogenic influenza strains such as H5N1, some manufacturers have modified the hemagglutinin gene sequence in order to reduce the pathogenicity of the influenza strain and to make
10 it avirulent and more easily produced in embryonic eggs or mammalian cell culture. Others also use reassortant influenza strains in which the genetic sequences for the hemagglutinin and neuraminidase proteins are cloned in a high-yielding low pathogenic influenza donor strain (A/PR/8/34; Quan F-S et al, 2007). While these methods may produce useful vaccines, they do not provide a solution to the need for
15 high-volume, low cost and fast production of vaccines in the scale necessary to meet the global need in a normal year, and would almost certainly be insufficient in the face of a pandemic.

[009] Using this reverse genetic technology, one might also need to mutate the genetic sequence of the HA protein to make it avirulent. For highly pathogenic
20 influenza strains, the production of whole virus vaccines either requires confinement procedures or the resulting vaccines do not exactly match the genetic sequence of the circulating virus. In the case of live-attenuated vaccines, there is still a risk that the administered vaccine can recombine with an influenza virus from the host, leading to a new influenza virus.

25 [0010] While this method maintains the antigenic epitope and post-translational modifications, there are a number of drawbacks to this method, including the risk of contamination due to the use of whole virus and variable yields depending on virus strain. Sub-optimal levels of protection may result from genetic heterogeneity in the virus due to its introduction into eggs. Other disadvantages includes extensive
30 planning for obtaining eggs, contamination risks due to chemicals used in purification, and long production times. Also, persons hypersensitive to egg proteins may not be eligible candidates for receiving the vaccine.

[0011] In the case of a pandemic, split vaccine production is limited by the need to adapt the strain for growth in eggs and the variable production yields achieved. Although this technology has been used for years for the production of seasonal vaccines, it can hardly respond in a reasonable timeframe to a pandemic and
5 worldwide manufacturing capacity is limited.

[0012] To avoid the use of eggs, influenza viruses have also been produced in mammalian cell culture, for example in MDCK or PERC.6 cells, or the like. Another approach is reverse genetics, in which viruses are produced by cell transformation with viral genes. These methods, however, also requires the use of whole virus as
10 well as elaborate methods and specific culture environments.

[0013] 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;
15 Johansson, 1999), viral vectors, and DNA vaccine constructs (Olsen et al., 1997).

[0014] Specifics of an influenza virus infection are well known. Briefly , the infectious cycle is initiated by the attachment of the virion surface HA protein to a sialic acid-containing cellular receptor (glycoproteins and glycolipids). The NA protein mediates processing of the sialic acid receptor, and virus penetration into the
20 cell depends on HA-dependent receptor-mediated endocytosis. In the acidic confines of internalized endosomes containing an influenza virion, the HA protein undergoes conformational changes that lead to fusion of viral and cell membranes and virus uncoating and M2-mediated release of MI proteins from nucleocapsid-associated ribonucleoproteins (RNPs), which migrate into the cell nucleus for viral RNA
25 synthesis. Antibodies to HA proteins prevent virus infection by neutralizing virus infectivity, whereas antibodies to NA proteins mediate their effect on the early steps of viral replication.

[0015] Crawford et al. (1999) disclose expression of influenza HA in baculovirus infected insect cells. The expressed proteins are described as being capable of
30 preventing lethal influenza disease caused by avian H5 and H7 influenza subtypes. Johansson et al. (1999) teach that baculovirus-expressed influenza HA and NA

proteins induce immune responses in animals superior to those induced by a conventional vaccine. Immunogenicity and efficacy of baculovirus- expressed hemagglutinin of equine influenza virus was compared to a homologous DNA vaccine candidate (Olsen et al., 1997). Collectively, these data demonstrate that a high
5 degree of protection against influenza virus challenge can be induced with recombinant HA or NA proteins, using various experimental approaches and in different animal models.

[0016] Since previous research has shown that the surface influenza glycoproteins, HA and NA, are the primary targets for elicitation of protective immunity against
10 influenza virus and that M1 provides a conserved target for cellular immunity to influenza, a new vaccine candidate may include these viral antigens as a protein macromolecular particle, such as virus-like particles (VLPs). As vaccine products, VLPs offer the advantage of being more immunogenic than subunit or recombinant antigens and are able to stimulate both humoral and cellular immune response
15 (Grgacic and Anderson, 2006). Further, the particle with these influenza antigens may display conformational epitopes that elicit neutralizing antibodies to multiple strains of influenza viruses.

[0017] Production of a non-infectious influenza virus strain for vaccine purposes is one way to avoid inadvertent infection. Alternatively, virus-like particles (VLPs) as
20 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.

[0018] Several studies have demonstrated that recombinant influenza proteins self-assemble into VLPs in cell culture using mammalian expression plasmids or
25 baculovirus vectors (Gomez-Puertas et al., 1999; Neumann et al., 2000; Latham and Galarza, 2001). Gomez-Puertas et al. (1999) discloses that efficient formation of influenza VLP depends on the expression levels of several viral proteins. Neumann et al. (2000) established a mammalian expression plasmid- based system for generating infectious influenza virus-like particles entirely from cloned cDNAs. Latham and
30 Galarza (2001) reported the formation of influenza VLPs in insect cells infected with recombinant baculovirus co-expressing HA, NA, M1, and M2 genes. These studies

demonstrated that influenza virion proteins may self-assemble upon co-expression in eukaryotic cells.

[0019] Gomez-Puertas et al.(2000) teach that, in addition to the hemagglutinin (HA), the matrix protein (M1) of the influenza virus is essential for VLP budding from insect cells. However, Chen et al. (2007) teach that M1 might not be required for VLP formation, and observed that efficient release of M1 and VLPs required the presence of HA and sialidase activity provided by NA. The NA cleaves the sialic acids of the glycoproteins at the surface of the cells producing the VLPs, and releasing the VLPs in the medium.

[0020] Quan et al 2007 teaches that a VLP vaccine produced in a baculovirus expression system (insect cell) induces a protective immunity against some strains of influenza virus (A/PR8/34 (H1N1)). The VLPs studied by Quan were observed to bud from the plasma membrane, and were considered to be of the correct size and morphology, similar to those obtained in a mammalian system (MDCK cells).

[0021] 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. Influenza virus particles and VLPs bud from the plasma membrane of the host cell. In mammalian or baculovirus cell systems, for example, influenza buds from the plasma membrane (Quan et al 2007). Only a few enveloped viruses are known to infect plants (for example, members of the Topoviruses 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.

[0022] 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, relying on the expression of only one viral protein is desirable to accelerate the development of vaccine.

[0023] In order to protect the world population from influenza and to stave off future pandemics, vaccine manufacturers will need to develop effective, rapid methods producing vaccine doses. The current use of fertilized eggs to produce vaccines is insufficient and involves a lengthy process.

5 SUMMARY OF THE INVENTION

[0024] It is an object of the invention to provide improved influenza virus like particles (VLPs).

[0025] According to the present invention there is provided a nucleic acid comprising a nucleotide sequence encoding an encoding an antigen from an enveloped virus
10 operatively linked to a regulatory region active in a plant. The antigen may be an influenza hemagglutinin (HA).

[0026] The present invention also provides a method of producing influenza virus like particles (VLPs) in a plant comprising:

- 15 a) introducing a nucleic acid encoding an antigen from an enveloped virus, for example an influenza hemagglutinin (HA), operatively linked to a regulatory region active in the plant, into the plant, or portion thereof, and
- b) incubating the plant or a portion thereof under conditions that permit the expression of the nucleic acid, thereby producing the VLPs.

[0027] The method may further comprise the steps of harvesting the plant and
20 purifying or separating the VLPs from the plant tissue.

[0028] The present invention includes the above method wherein, in the step of introducing (step a), the nucleic acid may be either transiently expressed in the plant, or stably expressed in the plant. Furthermore, the VLPs may be purified using size exclusion chromatography.

25 [0029] The present invention also provides a virus like particle (VLP) comprising an influenza virus HA protein and one or more than one plant lipid.

[0030] Also included in the present invention is a composition comprising an effective dose of a VLP comprising an influenza virus HA protein, one or more than one plant lipid, and a pharmaceutically acceptable carrier.

[0031] The present invention also contemplates fragments or portions of HA proteins that form VLPs in a plant.

[0032] The VLP may comprise an HA protein of one, or more than one subtype, including H1, H2, H3, H4, H5, H6, H7, H8, H9, H10, H11, H12, H13, H14, H15 or H16 or fragment or portion thereof. Examples of subtypes comprising such HA proteins include A/New Caledonia/20/99 (H1N1), A/Indonesia/5/2006 (H5N1), A/chicken/New York/1995, A/herring gull/DE/677/88 (H2N8), A/Texas/32/2003, A/mallard/MN/33/00, A/duck/Shanghai/1/2000, A/northern pintail/TX/828189/02, A/Turkey/Ontario/6118/68(H8N4), A/shoveler/Iran/G54/03, A/chicken/Germany/N/1949(H10N7), A/duck/England/56(H11N6), A/duck/Alberta/60/76(H12N5), A/Gull/Maryland/704/77(H13N6), A/Mallard/Gurjev/263/82, A/duck/Australia/341/83 (H15N8), A/black-headed gull/Sweden/5/99(H16N3), B/Lee/40, C/Johannesburg/66, A/PuertoRico/8/34 (H1N1), A/Brisbane/59/2007 (H1N1), A/Solomon Islands 3/2006 (H1N1), A/Brisbane 10/2007 (H3N2), A/Wisconsin/67/2005 (H3N2), B/Malaysia/2506/2004, B/Florida/4/2006, A/Singapore/1/57 (H2N2), A/Anhui/1/2005 (H5N1), A/Vietnam/1194/2004 (H5N1), A/Teal/HongKong/W312/97 (H6N1), A/Equine/Prague/56 (H7N7), A/HongKong/1073/99 (H9N2)).

[0033] In an aspect of the invention, the HA protein may be an H1, H2, H3, H5, H6, H7 or H9 subtype. In another aspect, the H1 protein may be from the A/New Caledonia/20/99 (H1N1), A/PuertoRico/8/34 (H1N1), A/Brisbane/59/2007 (H1N1), or A/Solomon Islands 3/2006 (H1N1) strain. The H3 protein may also be from the A/Brisbane 10/2007 (H3N2) or A/Wisconsin/67/2005 (H3N2) strain. In a further aspect of the invention, the H2 protein may be from the A/Singapore/1/57 (H2N2) strain. The H5 protein may be from the A/Anhui/1/2005 (H5N1), A/Vietnam/1194/2004 (H5N1), or A/Indonesia/5/2005 strain. In an aspect of the invention, the H6 protein may be from the A/Teal/HongKong/W312/97 (H6N1) strain. The H7 protein may be from the A/Equine/Prague/56 (H7N7) strain. In an aspect of the invention, the H9 protein is from the A/HongKong/1073/99 (H9N2)

strain. In a further aspect of the invention, the HA protein may be from an influenza virus may be a type B virus, including B/Malaysia/2506/2004 or B/Florida/4/2006. Examples of amino acid sequences of the HA proteins from H1, H2, H3, H5, H6, H7 or H9 subtypes include SEQ ID NOs: 48-59.

5 [0034] The influenza virus HA protein may be H5 Indonesia.

[0035] The present invention also provides nucleic acid molecules comprising sequences encoding an HA protein. The nucleic acid molecules may further comprise one or more regulatory regions operatively linked to the sequence encoding an HA protein.. The nucleic acid molecules may comprise a sequence encoding an H1, H2,
10 H3, H4, H5, H6, H7, H8, H9, H10, H11, H12, H13, H14, H15 or H16. In an aspect of the invention, the HA protein encoded by the nucleic acid molecule may be an H1, H2, H3, H5, H6, H7 or H9 subtype. The H1 protein encoded by the nucleic acid molecule is from the A/New Caledonia/20/99 (H1N1), A/PuertoRico/8/34 (H1N1), A/Brisbane/59/2007 (H1N1), or A/Solomon Islands 3/2006 (H1N1) strain. In an
15 aspect of the invention, the H3 protein encoded by the nucleic acid molecule may be from the A/Brisbane 10/2007 (H3N2), or A/Wisconsin/67/2005 (H3N2) strain. In a further aspect of the invention, the H2 protein encoded by the nucleic acid molecule may be from the A/Singapore/1/57 (H2N2) strain. The H5 protein encoded by the nucleic acid molecule may also be from the A/Anhui/1/2005 (H5N1),
20 A/Vietnam/1194/2004 (H5N1), or A/Indonesia/5/2005 strain. In an aspect of the invention, the H6 protein encoded by the nucleic acid molecule may be from the A/Teal/HongKong/W312/97 (H6N1) strain. The H7 protein encoded by the nucleic acid molecule may also be from the A/Equine/Prague/56 (H7N7) strain. Additionally, the H9 protein encoded by the nucleic acid molecule may be from the
25 A/HongKong/1073/99 (H9N2) strain. Examples of sequences of nucleic acid molecules encoding such HA proteins from H1, H2, H3, H5, H6, H7 or H9 subtypes include SEQ ID NOs: 36-47 and 60-73.

[0036] The nucleic acid sequence may encode the influenza virus HA protein H5 Indonesia.

30 [0037] Regulatory regions that may be operatively linked to a sequence encoding an 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), ST-LS1, a polyhedrin regulatory region, or a gp64 regulatory region. Other regulatory regions include a 5' UTR, 3' UTR or terminator sequences. The plastocyanin regulatory region may be an alfalfa plastocyanin regulatory region; the 5' UTR, 3'UTR or terminator sequences may also be alfalfa sequences.

[0038] A method of inducing immunity to an influenza virus infection in a subject, is also provided, the method comprising administering the virus like particle comprising an influenza virus HA protein, one or more than one plant lipid, and a pharmaceutically acceptable carrier. The virus like particle may be administered to a subject orally, intradermally, intranasally, intramuscularly, intraperitoneally, intravenously, or subcutaneously.

[0039] The present invention also pertains to a virus like particle (VLP) comprising one or more than one protein derived from a virus selected from the group consisting of Influenza, Measles, Ebola, Marburg, and HIV, and one or more than one lipid derived from a non-sialylating host production cell. The HIV protein may be p24, gp120 or gp41; the Ebolavirus protein may be VP30 or VP35; the Marburg virus protein may be Gp/SGP; the Measles virus protein may be H-protein or F-protein.

[0040] Additionally the present invention relates to a virus like particle (VLP) comprising an influenza virus HA protein and one or more than one host lipid. For example if the host is insect, then the virus like particle (VLP) may comprise an influenza virus HA protein and one or more than one insect lipid, or if the host is a yeast, then the virus like particle (VLP) may comprise an influenza virus HA protein and one or more than one yeast lipid.

[0041] The present invention also relates to compositions comprising VLPs of two or more strains or subtypes of influenza. The two or more subtypes or strains may be selected from the group comprising: A/New Caledonia/20/99 (H1N1)A/Indonesia/5/2006 (H5N1), A/chicken/New York/1995, A/herring gull/DE/677/88 (H2N8), A/Texas/32/2003, A/mallard/MN/33/00, A/duck/Shanghai/1/2000, A/northern pintail/TX/828189/02,

A/Turkey/Ontario/6118/68(H8N4), A/shoveler/Iran/G54/03,
 A/chicken/Germany/N/1949(H10N7), A/duck/England/56(H11N6),
 A/duck/Alberta/60/76(H12N5), A/Gull/Maryland/704/77(H13N6),
 A/Mallard/Gurjev/263/82, A/duck/Australia/341/83 (H15N8), A/black-headed
 5 gull/Sweden/5/99(H16N3), B/Lee/40, C/Johannesburg/66, A/PuertoRico/8/34
 (H1N1), A/Brisbane/59/2007 (H1N1), A/Solomon Islands 3/2006 (H1N1),
 A/Brisbane 10/2007 (H3N2), A/Wisconsin/67/2005 (H3N2), B/Malaysia/2506/2004,
 B/Florida/4/2006, A/Singapore/1/57 (H2N2), A/Anhui/1/2005 (H5N1),
 A/Vietnam/1194/2004 (H5N1), A/Teal/HongKong/W312/97 (H6N1),
 10 A/Equine/Prague/56 (H7N7) or A/HongKong/1073/99 (H9N2)). The two or more
 subtypes or strains of VLPs may be present in about equivalent quantities; alternately
 one or more of the subtypes or strains may be the majority of the strains or subtypes
 represented.

[0042] The present invention pertains to a method for inducing immunity to influenza
 15 virus infection in an animal or target organism comprising administering an effective
 dose of a vaccine comprising one or more than one VLP, the VLP produced using a
 non-sialating host, for example a plant host, an insect host, or a yeast host. The
 vaccine may be administered orally, intradermally, intranasally, intramuscularly,
 intraperitoneally, intravenously, or subcutaneously. The target organism may be
 20 selected from the group comprising 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, whales and the like.

[0043] The present invention provides a method for producing VLPs containing
 25 hemagglutinin (HA) from different influenza strains in a suitable host capable of
 producing a VLP, for example, a plant, insect, or yeast. VLPs that are produced in
 plants contain lipids of plant origin, VLPs produced in insect cells comprise lipids
 from the plasma membrane of insect cells (generally referred to as "insect lipids"),
 and VLPs produced in yeast comprise lipids from the plasma membrane of yeast
 30 cells (generally referred to as "yeast lipids").

[0044] 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 (Tsuji M., 2006). 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 (Saint-Jore-Dupas, 2007), may be advantageous of the production of VLPs in plants.

[0045] Without wishing to be bound by theory, it is anticipated that plant-made VLPs will induce a stronger immune reaction than VLPs made in other manufacturing systems and that the immune reaction induced by these plant-made VLPs will be stronger when compared to the immune reaction induced by live or attenuated whole virus vaccines.

[0046] Contrary to vaccines made of whole viruses, 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 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.

[0047] 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.

[0048] The VLPs produced in accordance with the present invention do not comprise M1 protein which is known to bind RNA. RNA is a contaminant of the VLP preparation and is undesired when obtaining regulatory approval for the VLP product.

[0049] This summary of the invention does not necessarily describe all features of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

5 [0050] 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:

[0051] FIGURE 1A shows a sequence of an alfalfa plastocyanin-based expression cassette used for the expression of H1 in accordance with an embodiment of the present invention (SEQ ID NO:8). Protein disulfide isomerase (PDI) signal peptide is underlined. BglII (AGATCT) and SacI (GAGCTC) restriction sites used for cloning are shown in bold. Figure 1B shows a schematic diagram of functional domains of influenza hemagglutinin. After cleavage of HA0, HA1 and HA2 fragments remain bound together by a disulfide bridge.

15 [0052] FIGURE 2A shows a representation of plasmid 540 assembled for the expression of HA subtype H1. FIGURE 2B shows a representation of plasmid 660 assembled for the expression of HA subtype H5.

[0053] FIGURE 3 shows a size exclusion chromatography of protein extracts from leaves producing hemagglutinin H1 or H5. FIGURE 3A show the elution profile of H1; Blue Dextran 2000 (triangles) and proteins (diamonds). FIGURE 3B shows immunodetection (western blot; anti H1) of H1 elution fractions following size exclusion chromatography (S500HR beads). FIGURE 3C show the elution profile of H5; Blue Dextran 2000 (triangles) and proteins (diamonds). FIGURE 3D shows immunodetection (western blot; anti H5) of H5 elution fractions following size exclusion chromatography (S500HR beads).

25 [0054] FIGURE 4 shows an electron microscopy photomicrograph of large hemagglutinin H1 and H5 structures from elution fraction 9 from a size exclusion column. FIGURE 4A shows a 50 000-fold enlargement of a VLP from H1 showing the presence of multiple similar structures (the bar represents 200 nm). FIGURE 4B shows a 150 000-fold enlargement of a VLP from H1 (the bar represents 100 nm). FIGURE 4C shows a 50 000-fold enlargement of a VLP from H5 showing the presence of multiple similar structures (the bar represents 50 nm).

30

[0055] FIGURE 5A shows the sequence of the N terminal fragment of H1 (SEQ ID NO:1). FIGURE 5B shows the C terminal fragment of H1 (SEQ ID NO:2). FIGURE 5C shows the complete sequence encoding HA0 of H1 (SEQ ID NO:28).

[0056] FIGURE 6 shows the sequence encoding H5 flanked by a HindIII site immediately upstream of the initial ATG, and a SacI site immediately downstream of the stop (TAA) codon (SEQ ID NO:3)

[0057] FIGURE 7A shows the sequence of the primer Plasto-443c (SEQ ID NO:4). FIGURE 7B shows the sequence of primer SpHA(Ind)-Plasto.r (SEQ ID NO:5). FIGURE 7C shows the sequence of primer Plasto-SpHA(Ind).c (SEQ ID NO:6). FIGURE 7D shows the sequence of primer HA(Ind)-Sac.r (SEQ ID NO:7).

[0058] FIGURE 8A shows the amino acid sequence of the HA1 peptide sequence (SEQ ID NO:9). FIGURE 8B shows the amino acid sequence of HA5 peptide sequence (SEQ ID NO:10). Native signal peptide is indicated in bold.

[0059] FIGURE 9 shows the sequence of HA of influenza A subtype H7 (SEQ ID No: 11).

[0060] FIGURE 10A shows the sequence of Influenza A HA, subtype H2 (SEQ ID NO:12). FIGURE 10B shows the sequence of Influenza A HA subtype H3 (SEQ ID NO:13). FIGURE 10C shows the sequence of Influenza A HA subtype H4 (SEQ ID NO:14). FIGURE 10D shows the sequence of Influenza A HA subtype H5 (SEQ ID NO:15). FIGURE 10E shows the sequence of Influenza A HA subtype H6 (SEQ ID NO:16). FIGURE 10F shows the sequence of Influenza A HA subtype H8 (SEQ ID NO:17). FIGURE 10G shows the sequence of Influenza A HA subtype H9 (SEQ ID NO:18). FIGURE 10H shows the sequence of Influenza A HA subtype H10 (SEQ ID NO:19). FIGURE 10I shows the sequence of Influenza A HA subtype H11 (SEQ ID NO:20). FIGURE 10J shows the sequence of Influenza A HA subtype H12 (SEQ ID NO:21). FIGURE 10K shows the sequence of Influenza A HA subtype H13 (SEQ ID NO:22). FIGURE 10L shows the sequence of Influenza A HA subtype H14 (SEQ ID NO:23). FIGURE 10M shows the sequence of Influenza A HA subtype H15 (SEQ ID NO:24). FIGURE 10N shows the sequence of Influenza A HA subtype H16 (SEQ ID NO:25). FIGURE 10O shows the sequence of Influenza B HA (SEQ ID NO:26).

FIGURE 10P shows the sequence of Influenza C HA (SEQ ID NO:27). FIGURE 10Q shows the sequence of primer XmaI-pPlas.c (SEQ ID NO: 29). FIGURE 10R shows the sequence of primer SacI-ATG-pPlas.r (SEQ ID NO: 30). FIGURE 10S shows the sequence of primer SacI-PlasTer.c (SEQ ID NO: 31). FIGURE 10T shows the sequence of primer EcoRI-PlasTer.r (SEQ ID NO: 32).

[0061] FIGURE 11 shows a schematic representation of several constructs as used herein. Construct 660 comprises the nucleotide sequence to encode the HA subtype H5 under operatively linked to the plastocyanin promoter (plasto) and terminator (Pter); construct 540 comprises the nucleotide sequence to encode the HA subtype H1 in combination with an alfalfa protein disulfide isomerase signal peptide (SP PDI), and is operatively linked to a plastocyanin promoter (Plasto) and terminator (Pter); construct 544 assembled for the expression of HA subtype H1, the nucleotide sequence encoding H1 is combined with an alfalfa protein disulfide isomerase signal peptide (SP PDI) and an GCN4pII leucine zipper (in place of the transmembrane domain and cytoplasmic tail of HI) and operatively linked to the plastocyanin promoter (Plasto) and terminator (Pter); and construct 750 for the expression of M1 coding region from influenza A/PR/8/34 is combined to the tobacco etch virus (TEV) 5'UTR, and operatively linked with the double 35S promoter and Nos terminator.

[0062] FIGURE 12 shows immunodetection of H5, using anti-H5 (Vietnam) antibodies, in protein extracts from *N. benthamiana* leaves transformed with construct 660 (lane 3). Commercial H5 from influenza A/Vietnam/1203/2004 was used as positive control of detection (lane 1), and a protein extract from leaves transformed with an empty vector were used as negative control (lane 2).

[0063] FIGURE 13 shows characterization of hemagglutinin structures by size exclusion chromatography. Protein extract from separate biomasses producing H5, H1, soluble H1, or H1 and M1 were separated by gel filtration on S-500 HR. Commercial H1 in the form of rosettes was also fractionated (H1 rosette). FIGURE 13A shows elution fractions analyzed for relative protein content (Relative Protein Level – a standard protein elution profile of a biomass fractionation is shown). Blue Dextran 2000 (2 MDa reference standard) elution peak is indicated. FIGURE 13B shows elution fractions analyzed for the presence of hemagglutinin by immunoblotting with anti-H5 (Vietnam) antibodies (for H5). FIGURE 13C shows

elution fractions analyzed for anti-influenza A antibodies for H1. FIGURE 13D shows elution fractions analyzed for anti-influenza A antibodies for soluble H1. FIGURE 13E shows elution fractions analyzed for anti-influenza A antibodies for H1 rosette. FIGURE 13F shows elution fractions analyzed for anti-influenza A antibodies for H1+M1.

[0064] FIGURE 14 shows concentration of influenza H5 structures by sucrose gradient centrifugation and electron microscopy examination of hemagglutinin-concentrated fractions. FIGURE 14A shows characterization of fractions from sucrose density gradient centrifugation. Each fraction was analyzed for the presence of H5 by immunoblotting using anti-H5 (Vietnam) antibodies (upper panel), and for their relative protein content and hemagglutination capacity (graph). FIGURE 14B shows negative staining transmission electron microscopy examination of pooled fractions 17, 18 and 19 from sucrose gradient centrifugation. The bar represents 100 nm.

[0065] FIGURE 15 shows purification of influenza H5 VLPs. FIGURE 15A shows Coomassie Blue stained SDS-PAGE analysis of protein content in the clarification steps – lane 1, crude extract; lane 2, pH 6-adjusted extract; lane 3, heat-treated extract; lane 4, DE-filtrated extract; the fetuin affinity purification steps: lane 5, load; lane 6, wash; lane 7, elution (10X concentrated). FIGURE 15B shows negative staining transmission electron microscopy examination of the purified H5 VLP sample. The bar represents 100 nm. FIGURE 15 C shows isolated H5 VLP enlarged to show details of the structure. FIGURE 15D shows the H5 VLP product on a Coomassie-stained reducing SDS-PAGE (lane A) and Western blot (lane B) using rabbit polyclonal antibody raised against HA from strain A/Vietnam/1203/2004 (H5N1).

[0066] FIGURE 16 shows a nucleotide sequence for Influenza A virus (A/New Caledonia/20/99(H1N1)) hemagglutinin (HA) gene, complete cds. GenBank Accession No. AY289929 (SEQ ID NO: 33)

[0067] FIGURE 17 shows a nucleotide sequence for *Medicago sativa* mRNA for protein disulfide isomerase. GenBank Accession No. Z11499 (SEQ ID NO: 34).

[0068] FIGURE 18 shows a nucleotide sequence for Influenza A virus (A/Puerto Rico/8/34(H1N1)) segment 7, complete sequence. GenBank Accession No. NC_002016.1 (SEQ ID NO: 35).

5 [0069] FIGURE 19 shows localization of VLP accumulation by positive staining transmission electron microscopy observation of H5 producing tissue. CW: cell wall, ch: chloroplast, pm: plasma membrane, VLP: virus-like particle. The bar represents 100 nm.

10 [0070] FIGURE 20 shows induction of serum antibody responses 14 days after boost in Balb/c mice vaccinated with plant-made influenza H5 VLP or recombinant soluble HA. FIGURE 20(A) Antibody responses of mice immunized through intramuscular injection. FIGURE 20(B) Antibody responses of mice immunized through intranasal administration. Antibody responses were measured against inactivated whole H5N1 viruses (A/Indonesia/5/05). GMT: geometric mean titer. Values are the GMT ($\log_2$) of reciprocal end-point titers of five mice per group. Bars represent *mean deviation*. * $p < 0.05$ compared to recombinant soluble HA.

15 [0071] FIGURE 21 shows hemagglutination inhibition antibody response (HAI) 14 days after boost in Balb/c mice vaccinated with plant-made influenza H5 VLP or recombinant soluble HA. FIGURE 21(A) Antibody responses of mice immunized through intramuscular injection. FIGURE 21(B) Antibody responses of mice immunized through intranasal administration. HAI antibody responses were measured using inactivated whole H5N1 viruses (A/Indonesia/5/05). GMT: geometric mean titer. Values are the GMT ($\log_2$) of reciprocal end-point titers of five mice per group. Bars represent *mean deviation*. * $p < 0.05$ and ** $p < 0.01$ compared to recombinant soluble HA.

25 [0072] FIGURE 22 shows the effect of adjuvant on immunogenicity of the VLPs in mice. FIGURE 22(A) Effect of alum on mice immunized through intramuscular injection FIGURE 22(B) Effect of Chitosan on mice immunized through intranasal administration. HAI antibody responses were measured using inactivated whole H5N1 viruses (A/Indonesia/5/05). GMT: geometric mean titer. Values are the GMT ($\log_2$) of reciprocal end-point titers of five mice per group. Bars represent *mean deviation*. * $p < 0.05$ compared to the corresponding recombinant soluble HA.

30

[0073] FIGURE 23 shows antibody response to VLP administration. FIGURE 23(A) Anti-Indonesia/5/05 immunoglobulin isotype in mice vaccinated with intramuscular injection, 30 days after boost. Values are the GMT ($\log_2$) of reciprocal end-point titers of five mice per group. ELISA performed using whole inactivated viruses as the coating agent. Bars represent *mean deviation*. * $p < 0.05$, ** $p < 0.001$ compared to the corresponding recombinant soluble HA. FIGURE 23(B) Antibody titers against whole inactivated viruses. All groups are statistically different to negative control.

[0074] FIGURE 24 shows antibody titer against homologous whole inactivated viruses (A/Indonesia/5/05), 2 weeks after first dose (week 2), 14 days after boost (week 5) or 30 days after boost (week 7). GMT: geometric mean titer. Values are the GMT ($\log_2$) of reciprocal end-point titers of five mice per group. * $p < 0.05$ compared to recombinant soluble HA.

[0075] FIGURE 25 shows in vitro cross-reactivity of serum antibodies. (A) Antibody titers against whole inactivated viruses. (B) Hemagglutination-inhibition titers against various whole inactivated viruses. Values are the GMT ($\log_2$) of reciprocal end-point titers of five mice per group. Bars represent *mean deviation*. All groups are statistically different to negative control. * $p < 0.05$, ** $p < 0.001$ compared to the corresponding recombinant soluble HA.

[0076] FIGURE 26 shows efficacy of the plant made H5 VLP. (A) Survival rate of mice after challenge with 10 LD₅₀ (4.09×10^5 CCID₅₀) of the influenza strain A/Turkey/582/06 (H5N1) (B) Body weight of immunised mice after challenge. Values are the mean body weight of surviving mice

[0077] FIGURE 27 shows Origin of plant-derived influenza VLPs. (A) Polar lipid composition of purified influenza VLPs. Lipids contained in an equivalent of 40 μg of proteins, were extracted from VLP as described, separated by HP-TLC, and compared to the migration profile of lipids isolated from highly purified tobacco plasma membrane (PM). Lipid abbreviations are as following: DGDG, Digalactosyldiacylglycerol; gluCER, glucosyl-ceramide; PA, phosphatic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine; SG, Steryl-glycoside. (B) Neutral lipid composition of purified influenza VLPs. Lipids contained in an equivalent of 20 μg of

proteins were extracted from VLP as described, separated by HP-TLC and compared to the migration of sitosterol. (C) Immunodetection of the plasma membrane marker proton pump ATPase (PMA) in purified VLPs and highly-purified PM from tobacco leaves (PM_L) and BY2 tobacco cells (PM_{BY2}). Eighteen micrograms of protein were loaded in each lane.

[0078] FIGURE 28 shows the sequence spanning from DraIII to SacI sites of clone 774 - nucleotide sequence of A/Brisbane/59/2007 (H1N1) (SEQ ID NO: 36) . The coding sequence is flanked by a plastocyanin regulatory region, starting with a DraIII restriction site at the 5' end and by a stop codon and a SacI site at the 3' end.

Restriction sites are underlined; ATG is in bold and underlined.

[0079] FIGURE 29 shows the sequence spanning from DraIII to SacI sites of clone 775 - nucleotide sequence of A/Solomon Islands 3/2006 (H1N1) (SEQ ID NO: 37) . The coding sequence is flanked by a plastocyanin regulatory region, starting with a DraIII restriction site at the 5' end and by a stop codon and a SacI site at the 3' end.

Restriction sites are underlined; ATG is in bold and underlined.

[0080] FIGURE 30 shows the sequence spanning from DraIII to SacI sites of clone 776 - nucleotide sequence of A/Brisbane 10/2007 (H1N1) (SEQ ID NO: 38) . The coding sequence is flanked by a plastocyanin regulatory region, starting with a DraIII restriction site at the 5' end and by a stop codon and a SacI site at the 3' end.

Restriction sites are underlined; ATG is in bold and underlined.

[0081] FIGURE 31 shows the sequence spanning from DraIII to SacI sites of clone 777 - nucleotide sequence of A/Wisconsin/67/2005 (H3N2) (SEQ ID NO: 39) . The coding sequence is flanked by a plastocyanin regulatory region, starting with a DraIII restriction site at the 5' end and by a stop codon and a SacI site at the 3' end.

Restriction sites are underlined; ATG is in bold and underlined.

[0082] FIGURE 32 shows the sequence spanning from DraIII to SacI sites of clone 778 - nucleotide sequence of B/Malaysia/2506/2004 (SEQ ID NO: 40) . The coding sequence is flanked by a plastocyanin regulatory region, starting with a DraIII restriction site at the 5' end and by a stop codon and a SacI site at the 3' end.

Restriction sites are underlined; ATG is in bold and underlined.

[0083] FIGURE 33 shows the sequence spanning from DraIII to SacI sites of clone 779 - nucleotide sequence of B/Florida/4/2006 (SEQ ID NO: 41) . The coding sequence is flanked by a plastocyanin regulatory region, starting with a DraIII restriction site at the 5' end and by a stop codon and a SacI site at the 3' end.

5 Restriction sites are underlined; ATG is in bold and underlined.

[0084] FIGURE 34 shows the sequence spanning from DraIII to SacI sites of clone 780 - nucleotide sequence of A/Singapore/1/57 (H2N2) (SEQ ID NO: 42) . The coding sequence is flanked by a plastocyanin regulatory region, starting with a DraIII restriction site at the 5' end and by a stop codon and a SacI site at the 3' end.

10 Restriction sites are underlined; ATG is in bold and underlined.

[0085] FIGURE 35 shows the sequence spanning from DraIII to SacI sites of clone 781 - nucleotide sequence of A/Anhui/1/2005 (H5N1) (SEQ ID NO: 43) . The coding sequence is flanked by a plastocyanin regulatory region, starting with a DraIII restriction site at the 5' end and by a stop codon and a SacI site at the 3' end.

15 Restriction sites are underlined; ATG is in bold and underlined.

[0086] FIGURE 36 shows the sequence spanning from DraIII to SacI sites of clone 782 - nucleotide sequence of A/Vietnam/1194/2004 (H5N1) (SEQ ID NO: 44) . The coding sequence is flanked by a plastocyanin regulatory region, starting with a DraIII restriction site at the 5' end and by a stop codon and a SacI site at the 3' end.

20 Restriction sites are underlined; ATG is in bold and underlined.

[0087] FIGURE 37 shows the sequence spanning from DraIII to SacI sites of clone 783 - nucleotide sequence of A/Teal/HongKong/W312/97 (H6N1) (SEQ ID NO: 45) . The coding sequence is flanked by a plastocyanin regulatory region, starting with a DraIII restriction site at the 5' end and by a stop codon and a SacI site at the 3' end.

25 Restriction sites are underlined; ATG is in bold and underlined.

[0088] FIGURE 38 shows the sequence spanning from DraIII to SacI sites of clone 784 - nucleotide sequence of A/Equine/Prague/56 (H7N7) (SEQ ID NO: 46) . The coding sequence is flanked by a plastocyanin regulatory region, starting with a DraIII restriction site at the 5' end and by a stop codon and a SacI site at the 3' end.

30 Restriction sites are underlined; ATG is in bold and underlined.

[0089] FIGURE 39 shows the sequence spanning from DraIII to SacI sites of clone 785 - nucleotide sequence of A/HongKong/1073/99 (H9N2) (SEQ ID NO: 47) . The coding sequence is flanked by a plastocyanin regulatory region, starting with a DraIII restriction site at the 5' end and by a stop codon and a SacI site at the 3' end.

5 Restriction sites are underlined; ATG is in bold and underlined.

[0090] FIGURE 40A shows the amino acid sequence (SEQ ID NO: 48) of the polypeptide translated from clone 774 (A/Brisbane/59/2007 (H1N1)). The open reading frame of clone 774 starts with the ATG indicated in Figure 28. Figure 40B shows the amino acid sequence (SEQ ID NO: 49) of the polypeptide translated from
10 clone 775 (A/Solomon Islands 3/2006 (H1N1)). The open reading frame of clone 775 starts with the ATG indicated in Figure 29.

[0091] FIGURE 41A shows the amino acid sequence (SEQ ID NO: 50) of the polypeptide translated from clone 776 (A/Brisbane/10/2007 (H3N2)). The open reading frame of clone 776 starts with the ATG indicated in Figure 30. Figure 41B
15 shows the amino acid sequence (SEQ ID NO: 51) of the polypeptide translated from clone 777 (A/Wisconsin/67/2005 (H3N2)). The open reading frame of clone 777 starts with the ATG indicated in Figure 31.

[0092] FIGURE 42A shows the amino acid sequence (SEQ ID NO: 52) of the polypeptide translated from clone 778 (B/Malaysia/2506/2004). The open reading
20 frame of clone 778 starts with the ATG indicated in Figure 32. Figure 42B shows the amino acid sequence (SEQ ID NO: 53) of the polypeptide translated from clone 779 (B/Florida/4/2006). The open reading frame of clone 779 starts with the ATG indicated in Figure 33.

[0093] FIGURE 43A shows the amino acid sequence (SEQ ID NO: 54) of the polypeptide translated from clone 780 (A/Singapore/1/57 (H2N2)). The open reading
25 frame of clone 780 starts with the ATG indicated in Figure 34. Figure 43B shows the amino acid sequence (SEQ ID NO: 55) of the polypeptide translated from clone 781 (A/Anhui/1/2005 (H5N1)). The open reading frame of clone 781 starts with the ATG indicated in Figure 35.

[0094] FIGURE 44A shows the amino acid sequence (SEQ ID NO: 56) of the polypeptide translated from clone 782 (A/Vietnam/1194/2004 (H5N1)). The open reading frame of clone 782 starts with the ATG indicated in Figure 36. Figure 44B shows the amino acid sequence (SEQ ID NO: 57) of the polypeptide translated from clone 783 (A/Teal/HongKong/W312/97 (H6N1)). The open reading frame of clone 783 starts with the ATG indicated in Figure 37.

[0095] FIGURE 45A shows the amino acid sequence (SEQ ID NO: 58) of the polypeptide translated from clone 784 (A/Equine/Prague/56 (H7N7)). The open reading frame of clone 784 starts with the ATG indicated in Figure 38. Figure 45B shows the amino acid sequence (SEQ ID NO: 59) of the polypeptide translated from clone 785 (A/HongKong/1073/99 (H9N2)). The open reading frame of clone 785 starts with the ATG indicated in Figure 39.

[0096] FIGURE 46 shows immunodetection (western blot) of elution fractions of plant-produced VLPs, following size exclusion chromatography. Hemagglutinin subtypes H1, H2, H5, H6 and H9 are shown. Hemagglutinin is detected in fractions 7-14, corresponding to the elution of VLPs.

[0097] FIGURE 47 shows an immunoblot analysis of expression of a series of H1 hemagglutinin from annual epidemic strains. Ten and twenty micrograms of protein extracts were loaded in lanes 1 and 2, respectively.

[0098] FIGURE 48 shows an immunoblot analysis of expression of a series of H5 hemagglutinin from potential pandemic strains. Ten and twenty micrograms of protein extracts were loaded in lanes 1 and 2, respectively.

[0099] FIGURE 49 show an immunoblot of H5 from strain A/Indonesia/5/2005 in protein extracts from *Nicotiana tabacum* leaves, agroinfiltrated with AGL1/660. Two plants were infiltrated and 10 and 20 µg of soluble protein from each plant were loaded in lanes 1 and 2, respectively.

[00100] FIGURE 50 shows the *in vitro* cross-reactivity of serum antibodies. Hemagglutination-inhibition (HI) titers in ferret sera, 14 days (A) after 1st immunization and (B) after 2nd boost with plant-made influenza H5 VLP. HAI antibody responses were measured using the following inactivated whole H5N1

viruses: A/turkey/Turkey/1/05, A/Vietnam/1194/04, A/Anhui/5/05 and the homologous strain A/Indonesia/5/05. Values are the GMT ($\log_2$) of reciprocal end-point titers of five ferrets per group. Diagonal stripe – A/Indonesia/6/06 (clade 2.1.3); checked – A/turkey/Turkey/1/05 (clade 2.2); white bar – A/Vietnam/1194/04 (clade 1); black bar A/Anhui/5/05. Responders are indicated. Bars represent *mean deviation*.

[00101] FIGURE 51 shows the nucleic acid sequence of an HA expression cassette comprising alfalfa plastocyanin promoter and 5' UTR, hemagglutinin coding sequence of H5 from A/Indonesia/5/2005 (Construct # 660), alfalfa plastocyanin 3' UTR and terminator sequences

[00102] FIGURE 52 shows the nucleic acid sequence of an HA expression cassette comprising alfalfa plastocyanin promoter and 5' UTR, hemagglutinin coding sequence of H1 from A/New Caledonia/20/1999 (Construct # 540), alfalfa plastocyanin 3' UTR and terminator sequences

[00103] FIGURE 53 shows the nucleic acid sequence of an HA expression cassette comprising alfalfa plastocyanin promoter and 5' UTR, hemagglutinin coding sequence of H1 from A/Brisbane/59/2007 (construct #774), alfalfa plastocyanin 3' UTR and terminator sequences.

[00104] FIGURE 54 shows the nucleic acid sequence of an HA expression cassette comprising alfalfa plastocyanin promoter and 5' UTR, hemagglutinin coding sequence of H1 from A/Solomon Islands/3/2006 (H1N1) (construct #775), alfalfa plastocyanin 3' UTR and terminator sequences.

[00105] FIGURE 55 shows the nucleic acid sequence of an HA expression cassette comprising alfalfa plastocyanin promoter and 5' UTR, hemagglutinin coding sequence of H2 from A/Singapore/1/57 (H2N2) (construct # 780), alfalfa plastocyanin 3' UTR and terminator sequences.

[00106] FIGURE 56 shows the nucleic acid sequence of an HA expression cassette comprising alfalfa plastocyanin promoter and 5' UTR, hemagglutinin coding sequence of H5 from A/Anhui/1/2005 (H5N1) (Construct# 781), alfalfa plastocyanin 3' UTR and terminator sequences

- [00107] FIGURE 57 shows the nucleic acid sequence of an HA expression cassette comprising alfalfa plastocyanin promoter and 5' UTR, hemagglutinin coding sequence of H5 from A/Vietnam/1194/2004 (H5N1) (Construct # 782), alfalfa plastocyanin 3' UTR and terminator sequences
- 5 [00108] FIGURE 58 shows the nucleic acid sequence of an HA expression cassette comprising alfalfa plastocyanin promoter and 5' UTR, hemagglutinin coding sequence of H6 from A/Teal/Hong Kong/W312/97 (H6N1) (Construct # 783), alfalfa plastocyanin 3' UTR and terminator sequences.
- 10 [00109] FIGURE 59 shows the nucleic acid sequence of an HA expression cassette comprising alfalfa plastocyanin promoter and 5' UTR, hemagglutinin coding sequence of H9 from A/Hong Kong/1073/99 (H9N2) (Construct # 785), alfalfa plastocyanin 3' UTR and terminator sequences.
- 15 [00110] FIGURE 60 shows the nucleic acid sequence of an HA expression cassette comprising alfalfa plastocyanin promoter and 5' UTR, hemagglutinin coding sequence of H3 from A/Brisbane/10/2007 (H3N2), alfalfa plastocyanin 3' UTR and terminator sequences.
- 20 [00111] FIGURE 61 shows the nucleic acid sequence of an HA expression cassette comprising alfalfa plastocyanin promoter and 5' UTR, hemagglutinin coding sequence of H3 from A/Wisconsin/67/2005 (H3N2), alfalfa plastocyanin 3' UTR and terminator sequences.
- 25 [00112] FIGURE 62 shows the nucleic acid sequence of an HA expression cassette comprising alfalfa plastocyanin promoter and 5' UTR, hemagglutinin coding sequence of H7 from A/Equine/Prague/56 (H7N7), alfalfa plastocyanin 3' UTR and terminator sequences.
- [00113] FIGURE 63 shows the nucleic acid sequence of an HA expression cassette comprising alfalfa plastocyanin promoter and 5' UTR, hemagglutinin coding sequence of HA from B/Malaysia/2506/2004, alfalfa plastocyanin 3' UTR and terminator sequences.

[00114] FIGURE 64 shows the nucleic acid sequence of an HA expression cassette comprising alfalfa plastocyanin promoter and 5' UTR, hemagglutinin coding sequence of HA from B/Florida/4/2006, alfalfa plastocyanin 3' UTR and terminator sequences.

5 [00115] FIGURE 65 shows a consensus amino acid sequence (SEQ ID NO: 74) for HA of A/New Caledonia/20/99 (H1N1) (encoded by SEQ ID NO: 33), A/Brisbane/59/2007 (H1N1) (SEQ ID NO: 48), A/Solomon Islands/3/2006 (H1N1) (SEQ ID NO: 49) and SEQ ID NO: 9. X1 (position 3) is A or V; X2 (position 52) is D or N; X3 (position 90) is K or R; X4 (position 99) is K or T; X5 (position 111) is Y
10 or H; X6 (position 145) is V or T; X7 (position 154) is E or K; X8 (position 161) is R or K; X9 (position 181) is V or A; X10 (position 203) is D or N; X11 (position 205) is R or K; X12 (position 210) is T or K; X13 (position 225) is R or K; X14 (position 268) is W or R; X15 (position 283) is T or N; X16 (position 290) is E or K; X17 (position 432) is I or L; X18 (position 489) is N or D.

15 [00116] FIGURE 66 shows Amino acid sequence of H1 New Caledonia (AAP34324.1) encoded by SEQ ID NO: 33.

[00117] FIGURE 67 shows the Amino acid sequence of H1 Puerto Rico (NC_0409878.1) encoded by SEQ ID NO: 35

20 DETAILED DESCRIPTION

[00118] The present invention relates to the production of virus-like particles. More specifically, the present invention is directed to the production of virus-like particles comprising influenza antigens.

[00119] The following description is of a preferred embodiment.

25 [00120] The present invention provides a nucleic acid comprising a nucleotide sequence encoding an antigen from an enveloped virus, for example, the influenza hemagglutinin (HA), operatively linked to a regulatory region active in a plant.

[00121] Furthermore, the present invention provides a method of producing virus like particles (VLPs) in a plant. The method involves introducing a nucleic acid
30 encoding an antigen 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 permit the expression of the nucleic acid, thereby producing the VLPs.

5 [00122] VLPs may be produced from influenza virus, however, VLPs may also be produced from other plasma membrane derived virus including but not limited to Measles, Ebola, Marburg, and HIV.

10 [00123] The invention includes VLPs of all types of influenza virus which may infect humans, including for example, but not limited to the very prevalent A (H1N1) sub-type (e.g. A/New Caledonia/20/99 (H1N1)), the A/Indonesia/5/05 sub-type (H5N1) (SEQ ID NO: 60) and the less common B type (for example SEQ ID NO:26, Figure 10O), and C type (SEQ ID NO:27, Figure 10P), and to HAs obtained from other influenza subtypes. VLPs of other influenza subtypes are also included in the present invention, for example, A/Brisbane/59/2007 (H1N1; SEQ ID NO:48), A/Solomon Islands/3/2006 (H1N1; SEQ ID NO:49), A/Singapore/1/57 (H2N2; SEQ ID NO:54), A/Anhui/1/2005 (H5N1; SEQ ID NO:55), A/Vietnam/1194/2004 (H5N1; SEQ ID NO:56), A/Teal/Hong Kong/W312/97 (H6N1; SEQ ID NO:57), A/Hong Kong/1073/99 (H9N2; SEQ ID NO:59), A/Brisbane/10/2007 (H3N2; SEQ ID NO:50), A/Wisconsin/67/2005 (H3N2; SEQ ID NO:51), A/Equine/Prague/56 (H7N7; SEQ ID NO:58), B/Malaysia/2506/2004 (SEQ ID NO:52), or B/Florida/4/2006 (SEQ ID NO:53).

25 [00124] 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.

30 [00125] Non limiting examples of other antigens that may be expressed in plasma membrane derived viruses include, the Capsid protein of HIV - p24; gp120, gp41 - envelope proteins, the structural proteins VP30 and VP35; Gp/SGP (a glycosylated integral membrane protein) of Filoviruses, for example Ebola or Marburg, or the H protein, and F protein of Paramyxoviruses, for example, Measles.

[00126] The invention also includes, but is not limited to, influenza derived VLPs that obtain a lipid envelope from the plasma membrane of the cell in which the VLP proteins are expressed. For example, if the VLP is expressed in a plant-based system, the VLP may obtain a lipid envelope from the plasma membrane of the cell.

5 [00127] Generally, the term “lipid” refers to a fat-soluble (lipophilic), naturally-occurring molecules. The term is also used more specifically to refer to fatty-acids and their derivatives (including tri-, di-, and monoglycerides and phospholipids), as well as other fat-soluble sterol-containing metabolites or sterols. Phospholipids are a major component of all biological membranes, along with
10 glycolipids, sterols and proteins. Examples of phospholipids include phosphatidylethanolamine, phosphatidylcholine, phosphatidylinositol, phosphatidylserine, and the like. Examples of sterols include zoosterols (e.g., cholesterol) and phytosterols. Over 200 phytosterols have been identified in various plant species, the most common being campesterol, stigmasterol, ergosterol,
15 brassicasterol, delta-7-stigmasterol, delta-7-avenasterol, daunosterol, sitosterol, 24-methylcholesterol, cholesterol or beta-sitosterol. As one of skill in the art would understand, the lipid composition of the plasma membrane of a cell may vary with the culture or growth conditions of the cell or organism from which the cell is obtained.

[00128] Cell membranes generally comprise lipid bilayers, as well as proteins
20 for various functions. Localized concentrations of particular lipids may be found in the lipid bilayer, referred to as ‘lipid rafts’. Without wishing to be bound by theory, lipid rafts may have significant roles in endo and exocytosis, entry or egress of viruses or other infectious agents, inter-cell signal transduction, interaction with other structural components of the cell or organism, such as intracellular and extracellular
25 matrices.

[00129] With reference to influenza virus, the term “hemagglutinin” or “HA” as used herein refers to a glycoprotein found on the outside of influenza viral particles. HA is a homotrimeric membrane type I glycoprotein, generally comprising a signal peptide, an HA1 domain, and an HA2 domain comprising a membrane-
30 spanning anchor site at the C-terminus and a small cytoplasmic tail (Figure 1B). Nucleotide sequences encoding HA are well known and are available – see, for example, the BioDefence Public Health base (Influenza Virus; see URL:

biohealthbase.org) or National Center for Biotechnology Information (see URL: ncbi.nlm.nih.gov), both of which are incorporated herein by reference.

[00130] The term “homotrimer” or “homotrimeric” indicates that an oligomer is formed by three HA protein molecules. Without wishing to be bound by theory, HA protein is synthesized as monomeric precursor protein (HA0) of about 75 kDa, which assembles at the surface into an elongated trimeric protein. Before trimerization occurs, the precursor protein is cleaved at a conserved activation cleavage site (also referred to as fusion peptide) into 2 polypeptide chains, HA1 and HA2 (comprising the transmembrane region), linked by a disulfide bond. The HA1 segment may be 328 amino acids in length, and the HA2 segment may be 221 amino acids in length. Although this cleavage may be important for virus infectivity, it may not be essential for the trimerization of the protein. Insertion of HA within the endoplasmic reticulum (ER) membrane of the host cell, signal peptide cleavage and protein glycosylation are co-translational events. Correct refolding of HA requires glycosylation of the protein and formation of 6 intra-chain disulfide bonds. The HA trimer assembles within the cis- and trans-Golgi complex, the transmembrane domain playing a role in the trimerization process. The crystal structures of bromelain-treated HA proteins, which lack the transmembrane domain, have shown a highly conserved structure amongst influenza strains. It has also been established that HA undergoes major conformational changes during the infection process, which requires the precursor HA0 to be cleaved into the 2 polypeptide chains HA1 and HA2. The HA protein may be processed (i.e., comprise HA1 and HA2 domains), or may be unprocessed (i.e. comprise the HA0 domain).

[00131] The present invention pertains to the use of an HA protein comprising the transmembrane domain and includes HA1 and HA2 domains, for example the HA protein may be HA0, or processed HA comprising HA1 and HA2. The HA protein may be used in the production or formation of VLPs using a plant, or plant cell, expression system.

[00132] The HA of the present invention may be obtained from any subtype. For example, the HA may be of subtype H1, H2, H3, H4, H5, H6, H7, H8, H9, H10, H11, H12, H13, H14, H15, or H16. The recombinant HA of the present invention may also comprise an amino acid sequence based on the sequence any hemagglutinin

known in the art— see, for example, the BioDefence Public Health base (Influenza Virus; see URL: biohealthbase.org) or National Center for Biotechnology Information (see URL: ncbi.nlm.nih.gov). Furthermore, the HA may be based on the sequence of a hemagglutinin that is isolated from one or more emerging or newly-identified influenza viruses.

[00133] The present invention also includes VLPs that comprise HAs obtained from one or more than one influenza subtype. For example, VLPs may comprise one or more than one HA from the subtype H1 (encoded by SEQ ID NO:28), H2 (encoded by SEQ ID NO:12), H3 (encoded by SEQ ID NO:13), H4 (encoded by SEQ ID NO:14), H5 (encoded by SEQ ID NO:15), H6 (encoded by SEQ ID NO:16), H7 (encoded by SEQ ID NO:11), H8 (encoded by SEQ ID NO:17), H9 (encoded by SEQ ID NO:18), H10 (encoded by SEQ ID NO:19), H11 (encoded by SEQ ID NO:20), H12 (encoded by SEQ ID NO:21), H13 (encoded by SEQ ID NO:27), H14 (encoded by SEQ ID NO:23), H15 (encoded by SEQ ID NO:24), H16 (encoded by SEQ ID NO:25), or a combination thereof. One or more than one HA from the one or more than one influenza subtypes may be co-expressed within a plant or insect cell to ensure that the synthesis of the one or more than one HA results in the formation of VLPs comprising a combination of HAs obtained from one or more than one influenza subtype. Selection of the combination of HAs may be determined by the intended use of the vaccine prepared from the VLP. For example a vaccine for use in inoculating birds may comprise any combination of HA subtypes, while VLPs useful for inoculating humans may comprise subtypes one or more than one of subtypes H1, H2, H3, H5, H7, H9, H10, N1, N2, N3 and N7. However, other HA subtype combinations may be prepared depending upon the use of the inoculum.

[00134] Therefore, the present invention is directed to a VLP comprising one or more than one HA subtype.

[00135] The present invention also provides for nucleic acids encoding hemagglutinins that form VLPs when expressed in plants

[00136] Influenza HA proteins exhibit a range of similarities and differences with respect to molecular weight, isoelectric point, size, glycan complement and the like. The physico-chemical properties of the various hemagglutinins may be useful to

allow for differentiation between the HAs expressed in a plant, insect cell or yeast system, and may be of particular use when more than one HA is co-expressed in a single system. Examples of such physico-chemical properties are provided in Table 1.

V81270WO

Table 1: Physico-chemical properties of influenza hemagglutinins

Clone No	Type	Influenza strains	AA			Glycans			Molecular Weight (kDA)						Isoelectric point		
			HA0	HA1	HA2	HA0	HA1	HA2	HA0	HA1	HA1 ₁	HA1 ₁	HA2 ₁	HA2 ₁	HA0	HA1	HA2
774	H1	A/Brisbane/59/2007	548	326	222	9	7	2	61	75	36	47	25	28	6.4	7.5	5.3
		A/Solomon															
775	H1	Islands/3/2006	548	326	222	9	7	2	61	75	36	47	25	28	6.1	6.7	5.3
776	H3	A/Brisbane/10/2007	550	329	221	12	11	1	62	80	37	54	25	27	8.5	9.6	5.2
777	H3	A/Wisconsin/67/2005	550	329	221	11	10	1	62	79	37	52	25	27	8.8	9.6	5.3
778	B	B/Malaysia/2506/2004	570	347	223	12	8	4	62	80	38	50	24	30	8.0	9.7	4.5
779	B	B/Florida/4/2006	569	346	223	10	7	3	62	77	38	48	24	29	8.0	9.7	4.5
780	H2	A/Singapore/1/57	547	325	222	6	4	2	62	71	36	42	25	28	6.0	7.5	4.9
781	H5	A/Anhui/1/2005	551	329	222	7	5	2	62	73	37	45	25	28	6.2	8.9	4.7
782	H5	A/Vietnam/1194/2004	552	330	222	7	5	2	63	74	38	45	25	28	6.4	9.1	4.8
		A/Teal/Hong Kong/W312/97															
783	H6	Kong/W312/97	550	328	222	8	5	3	62	75	37	45	25	30	5.7	5.9	5.6
784	H7	A/Equine/Prague/56	552	331	221	6	4	2	62	71	37	43	25	28	8.9	9.7	4.9
		A/Hong Kong/1073/99															
785	H9	Kong/1073/99	542	320	199	9	7	2	61	75	36	46	23	26	8.4	9.5	5.3

[00137] The present invention also includes nucleotide sequences SEQ ID NO:28; SEQ ID NO:3; SEQ ID NO:11, encoding HA from H1, H5 or H7, respectively, a nucleotide sequence that hybridizes under stringent hybridisation conditions to SEQ ID NO:28; SEQ ID NO:3; SEQ ID NO:11, , or a nucleotide sequence that hybridizes under stringent hybridisation conditions to a compliment of SEQ ID NO:28; SEQ ID NO:3; SEQ ID NO:1, wherein the nucleotide sequence encodes a hemagglutinin protein that when expressed forms a VLP, and that the VLP induces the production of an antibody when administered to a subject. For example, expression of the nucleotide sequence within a plant cell forms a VLP, and the VLP may be used to produce an antibody that is capable of binding HA, including mature HA, HA0, HA1, or HA2 of one or more influenza types or subtypes. The VLP, when administered to a subject, induces an immune response.

[00138] 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.

[00139] Additionally, the present invention includes nucleotide sequences that are characterized as having about 70, 75, 80, 85, 87, 90, 91, 92, 93 94, 95, 96, 97, 98, 99, 100% or any amount therebetween, sequence identity, or sequence similarity, with the nucleotide sequence encoding HA from H1 (SEQ ID NO:28), H5 (SEQ ID NO:3) or H7 (SEQ ID NO:11), wherein the nucleotide sequence encodes a hemagglutinin

protein that when expressed forms a VLP, and that the VLP induces the production of an antibody. For example, expression of the nucleotide sequence within a plant cell forms a VLP, and the VLP may be used to produce an antibody that is capable of binding HA, including mature HA, HA0, HA1, or HA2. The VLP, when
5 administered to a subject, induces an immune response.

[00140] Similarly, the present invention includes HAs associated with the following subtypes H1 (encoded by SEQ ID NO:28), H2 (encoded by SEQ ID NO:12), H3 (encoded by SEQ ID NO:13), H4 (encoded by SEQ ID NO:14), H5 (encoded by SEQ ID NO:15), H6 (encoded by SEQ ID NO:16), H7 (encoded by SEQ ID NO:11), H8 (encoded by SEQ ID NO:17), H9 (encoded by SEQ ID NO:18), H10 (encoded by SEQ ID NO:19), H11 (encoded by SEQ ID NO:20), H12 (encoded by SEQ ID NO:21), H13 (encoded by SEQ ID NO:27), H14 (encoded by SEQ ID NO:23), H15 (encoded by SEQ ID NO:24), H16 (encoded by SEQ ID NO:25); see
10 Figures 10A to 10P), and nucleotide sequences that are characterized as having from about 70 to 100% or any amount therebetween, 80 to 100% or any amount therebetween, 90-100% or any amount therebetween, or 95-100% or any amount therebetween, sequence identity with H1 (SEQ ID NO:28), H2 (SEQ ID NO:12), H3 (SEQ ID NO:13), H4 (SEQ ID NO:14), H5 (SEQ ID NO:15), H6 (SEQ ID NO:16), H7 (SEQ ID NO:11), H8 (SEQ ID NO:17), H9 (SEQ ID NO:18), H10 (SEQ ID NO:19), H11 (SEQ ID NO:20), H12 (SEQ ID NO:21), H13 (SEQ ID NO:27), H14 (SEQ ID NO:23), H15 (SEQ ID NO:24), H16 (SEQ ID NO:25), wherein the
15 nucleotide sequence encodes a hemagglutinin protein that when expressed forms a VLP, and that the VLP induces the production of an antibody. For example, expression of the nucleotide sequence within a plant cell forms a VLP, and the VLP may be used to produce an antibody that is capable of binding HA, including mature HA, HA0, HA1, or HA2. The VLP, when administered to a subject, induces an
20 immune response.

[00141] An “immune response” generally refers to a response of the adaptive immune system. The adaptive immune system generally comprises a humoral
30 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.

[00142] 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.

[00143] 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.

[00144] A hemagglutination inhibition (HI, or HAI) assay may also be used to demonstrate the efficacy of antibodies induced by a vaccine, or vaccine composition 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 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.

[00145] Cross-reactivity HAI titres may also be used to demonstrate the efficacy of an immune response to other strains of virus related to the vaccine

subtype. For example, serum from a subject immunized with a vaccine composition of a first strain (e.g. VLPs of A/Indonesia 5/05) may be used in an HAI assay with a second strain of whole virus or virus particles (e.g. A/Vietnam/1194/2004), and the HAI titer determined.

5 [00146] 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
10 by fluorescence-activated cell sorting (FACS), using marker specific fluorescent labels and methods as are known in the art.

[00147] 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
15 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)

[00148] Sequence identity or sequence similarity may be determined using a nucleotide sequence comparison program, such as that provided within DNASIS (for
20 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,
25 Proc. Nat'l. Acad. Sci. USA 85:2444), and by computerized implementations of these algorithms (e.g. GAP, BESTFIT, FASTA, and BLAST)., or by manual alignment and visual inspection.

[00149] The term "hemagglutinin domain" refers to a peptide comprising either the HA0 domain, or the HA1 and HA2 domains. The hemagglutinin domain does not
30 include the signal peptide, transmembrane domain, or the cytoplasmic tail found in the naturally occurring protein.

[00150] 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. VLPs are generally morphologically and antigenically similar to virions produced in an infection, but lack genetic information sufficient to replicate and thus are non-infectious. In some examples, VLPs may comprise a single protein species, or more than one protein species. For VLPs comprising more than one protein species, the protein species may be from the same species of virus, or may comprise a protein from a different species, genus, subfamily or family of virus (as designated by the ICTV nomenclature). In other examples, one or more of the protein species comprising a VLP may be modified from the naturally occurring sequence. VLPs may be produced in suitable host cells including plant and insect host cells. Following extraction from the host cell and upon isolation and further purification under suitable conditions, VLPs may be purified as intact structures.

[00151] The VLPs produced from influenza derived proteins, in accordance with the present invention do not comprise M1 protein. The M1 protein is known to bind RNA (Wakefield and Brownlee, 1989) which is a contaminant of the VLP preparation. The presence of RNA is undesired when obtaining regulatory approval for the VLP product, therefore a VLP preparation lacking RNA may be advantageous.

[00152] The VLPs of the present invention may be produced in a host cell that is characterized by lacking the ability to sialylate proteins, for example lacking sialidase, such as 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, as identified in glycoforum (see, for example, the URL: glycoforum.gr.jp/science/word/evolution/ES-A03E.html). The 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.

[00153] A VLP produced in a plant according to some aspects of the invention may be complexed with plant-derived lipids. The VLP may comprise an HA0, HA1 or HA2 peptide. 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, but not limited to, phosphatidylcholine (PC), phosphatidylethanolamine (PE), glycosphingolipids, phytosterols or a combination thereof. A plant-derived lipid may alternately be referred to as a 'plant lipid'.

5 Examples of phytosterols are known in the art, and include, for example, stigmasterol, sitosterol, 24-methylcholesterol and cholesterol – see, for example, Mongrand et al., 2004.

[00154] VLPs may be assessed for structure and size by, for example, hemagglutination assay, electron microscopy, or by size exclusion chromatography.

10 [00155] 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 Sephacryl™ column. Blue Dextran 2000 may be used as
15 a calibration standard. Following chromatography, fractions may be further analyzed by immunoblot to determine the protein complement of the fraction.

[00156] 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 $\alpha 2,3$ or $\alpha 2,6$ and the presence of these sialic acids on the surface of RBC. Equine and avian
20 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 (see also Ito T. et al, 1997, Virology, vol 227, p493-499; and Medeiros R et al, 2001, Virology, vol 289 p.74-85). Examples of the species reactivity of HAs of
25 different influenza strains is shown in Tables 2A and 2B.

Table 2A: Species of RBC bound by HAs of selected seasonal influenza strains.

Seasonal	Strain	No	Origin	Horse	Turkey
H1	A/Brisbane/59/2007 (H1N1)	774	Human	+	++
	A/Solomon Islands/3/2006 (H1N1)	775	Human	+	++
H3	A/Brisbane/10/2007 (H3N2)	776	Human	+	++
	A/Wisconsin/67/2005 (H3N2)	777	Human	+	++
B	B/Malaysia/2506/2004	778	Human	+	++
	B/Florida/4/2006	779	Human	+	++

Table 2B: Species of RBC bound by HAs of selected pandemic influenza strains

Pandemic	Strain	No	Origin	Horse	Turkey
H2	A/Singapore/1/57 (H2N2)	780	Human	+	++
H5	A/Anhui/1/2005 (H5N1)	781	Hu-Av	++	+
	A/Vietnam/1194/2004 (H5N1)	782	Hu-Av	++	+
H6	A/Teal/Hong Kong/W312/97 (H6N1)	783	Avian	++	+
H7	A/Equine/Prague/56 (H7N7)	784	Equine	++	++
H9	A/Hong Kong/1073/99 (H9N2)	785	Human	++	+

5 [00157] As used herein, a "protein" refers generally to a string of amino acids connected by a peptide bond, which may be folded into secondary, tertiary or quaternary structure to achieve a particular morphology. Alternately, the terms polypeptide, peptide or peptide fragments may be used in a similar context.

[00158] A fragment or portion of a protein, fusion protein or polypeptide
10 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 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
15 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.

[00159] 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 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 HA, and provided that the fragment can form a 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 HA, and provided that the fragment can form a 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 an HA protein, provided that the fragment can form a VLP when expressed.

[00160] 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 when constructing a clone for crystallography, 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.

[00161] Comparisons of a sequence or sequences may be done using a BLAST algorithm (Altschul et al., 1990. J. Mol Biol 215:403-410). A BLAST search allows for comparison of a query sequence with a specific sequence or group of sequences, or with a larger library or database (e.g. GenBank or GenPept) of sequences, and identify not only sequences that exhibit 100% identity, but also those with lesser degrees of identity. Nucleic acid or amino acid sequences may be compared using a BLAST algorithm. Furthermore the identity between two or more sequences may be determined by aligning the sequences together and determining the % identity between the sequences. Alignment may be carried out using the BLAST Algorithm

(for example as available through GenBank; URL: ncbi.nlm.nih.gov/cgi-bin/BLAST/ using default parameters: Program: blastn; Database: nr; Expect 10; filter: default; Alignment: pairwise; Query genetic Codes: Standard(1)), or BLAST2 through EMBL URL: embl-heidelberg.de/Services/index.html using default parameters: Matrix BLOSUM62; Filter: default, echofilter: on, Expect:10, cutoff: default; Strand: both; Descriptions: 50, Alignments: 50; or FASTA, using default parameters), or by manually comparing the sequences and calculating the % identity.

[00162] The present invention describes, but is not limited to, the cloning of a nucleic acid encoding HA into a plant expression vector, and the production of influenza VLPs from the plant, suitable for vaccine production. Examples of such nucleic acids include, for example, but are not limited to, an influenza A/New Caledonia/20/99 (H1N1) virus HA (e.g. SEQ ID NO: 61), an HA from A/Indonesia/5/05 sub-type (H5N1) (e.g. SEQ ID NO: 60), A/Brisbane/59/2007 (H1N1) (e.g. SEQ ID NO: 36, 48, 62), A/Solomon Islands/3/2006 (H1N1) (e.g. SEQ ID NO: 37, 49, 63), A/Singapore/1/57 (H2N2) (e.g. SEQ ID NO: 42, 54, 64), A/Anhui/1/2005 (H5N1) (e.g. SEQ ID NO: 43, 55, 65), A/Vietnam/1194/2004 (H5N1) (e.g. SEQ ID NO: 44, 56, 66), A/Teal/Hong Kong/W312/97 (H6N1) (e.g. SEQ ID NO: 45, 57, 67), A/Hong Kong/1073/99 (H9N2) (e.g. SEQ ID NO: 47, 59, 68), A/Brisbane/10/2007 (H3N2) (e.g. SEQ ID NO: 38, 50, 69), A/Wisconsin/67/2005 (H3N2) (e.g. SEQ ID NO: 39, 51, 70), A/Equine/Prague/56 (H7N7) (e.g. SEQ ID NO: 46, 58, 71), B/Malaysia/2506/2004 (e.g. SEQ ID NO: 40, 52, 72), B/Florida/4/2006 (e.g. SEQ ID NO: 41, 53, 73). The corresponding clone or construct numbers for these strains is provided in Table 1. Nucleic acid sequences corresponding to SEQ ID NOs: 36-47 comprise a plastocyanin upstream and operatively linked to the coding sequence of the HA for each of the types or subtypes, as illustrated in Figures 28-39. Nucleic acid sequences corresponding to SEQ ID NO: 60-73 comprise an HA expression cassette comprising alfalfa plastocyanin promoter and 5' UTR, hemagglutinin coding sequence of an HA, alfalfa plastocyanin 3' UTR and terminator sequences, as illustrated in Figures 51-64.

[00163] The VLPs may also be used to produce reagents comprised of recombinant influenza structural proteins that self-assemble into functional and immunogenic homotypic macromolecular protein structures, including subviral

influenza particles and influenza VLP, in transformed hosts cells, for example plant cells or insect cells.

[00164] Therefore, the invention provides for VLPs, and a method for producing viral VLPs in a plant expression system, from the expression of a single envelope protein. The VLPs may be influenza VLPs, or VLPs produced from other plasma membrane-derived virus including, but not limited to, Measles, Ebola, Marburg, and HIV.

[00165] Proteins from other enveloped viruses, for example but not limited to Filoviridae (e.g. Ebola virus, Marburg virus, or the like), Paramyxoviridae (e.g. Measles virus, Mumps virus, Respiratory syncytial virus, pneumoviruses, or the like), Retroviridae (e.g. Human Immunodeficiency Virus-1, Human Immunodeficiency Virus-2, Human T-Cell Leukemia Virus-1, or the like), Flaviviridae (e.g. West Nile Encephalitis, Dengue virus, Hepatitis C virus, yellow fever virus, or the like), Bunyaviridae (e.g. Hantavirus or the like), Coronaviridae (e.g. coronavirus, SARS, or the like), as would be known to those of skill in the art, may also be used. Non limiting examples of antigens that may be expressed in plasma membrane derived viruses include, the capsid protein of HIV - p24; HIV glycoproteins gp120 or gp41, Filovirus proteins including VP30 or VP35 of Ebolavirus or Gp/SGP of Marburg virus or the H protein or F protein of the Measles paramyxovirus. For example, P24 of HIV (e.g. GenBank reference gi:19172948) is the protein obtained by translation and cleavage of the *gag* sequence of the HIV virus genome (e.g. GenBank reference gi:9629357); gp 120 and gp41 of HIV are glycoproteins obtained by translation and cleavage of the gp160 protein (e.g. GenBank reference gi:9629363), encoded by *env* of the HIV virus genome. VP30 of Ebolavirus (GenPept Reference gi: 55770813) is the protein obtained by translation of the *vp30* sequence of the Ebolavirus genome (e.g. GenBank Reference gi:55770807) ; VP35 of Ebolavirus (GenPept Reference gi:55770809) is the protein obtained by translation of the *vp35* sequence of the Ebolavirus genome. Gp/SGP of Marburg virus (GenPept Reference gi:296965) is the protein obtained by translation of the (sequence) of the Marburg virus genome (GenBank Reference gi:158539108). H protein (GenPept Reference gi: 9626951) is the protein of the H sequence of the Measles virus genome (GenBank Reference gi:

9626945); F protein (GenPept reference gi: 9626950) is the protein of the F sequence of the Measles virus genome.

[00166] However, other coat proteins may be used within the methods of the present invention as would be known to one of skill in the art.

5 [00167] The invention, therefore, provides for a nucleic acid molecule comprising a sequence encoding HIV-p24, HIV-gp120, HIV-gp41, Ebolavirus-VP30, Ebolavirus-VP35, Marburg virus Gp/SGP, Measles virus-H protein or -F protein. The nucleic acid molecule may be operatively linked to a regulatory region active in an insect, yeast or plant cell, or in a particular plant tissue.

10 [00168] The present invention further provides the cloning of a nucleic acid encoding an HA, for example but not limited to, human influenza A/Indonesia/5/05 virus HA (H5N1) into a plant or insect expression vector (e.g. baculovirus expression vector) and production of influenza vaccine candidates or reagents comprised of recombinant influenza structural proteins that self-assemble into functional and
15 immunogenic homotypic macromolecular protein structures, including subviral influenza particles and influenza VLP, in transformed plant cells or transformed insect cells.

[00169] The nucleic acid encoding the HA of influenza subtypes, for example but not limited to, A/New Caledonia/20/99 (H1N1), A/Indonesia/5/05 sub-type
20 (H5N1), A/Brisbane/59/2007 (H1N1), A/Solomon Islands/3/2006 (H1N1), A/Singapore/1/57 (H2N2), A/Anhui/1/2005 (H5N1), A/Vietnam/1194/2004 (H5N1), A/Teal/Hong Kong/W312/97 (H6N1), A/Hong Kong/1073/99 (H9N2), A/Brisbane/10/2007 (H3N2), A/Wisconsin/67/2005 (H3N2), A/Equine/Prague/56 (H7N7), B/Malaysia/2506/2004, B/Florida/4/2006 may be expressed, for example,
25 using a Baculovirus Expression System in an appropriate cell line, for example, *Spodoptera frugiperda* cells (e.g. Sf-9 cell line; ATCC PTA-4047). Other insect cell lines may also be used.

[00170] The nucleic acid encoding the HA may, alternately, be expressed in a plant cell, or in a plant. The nucleic acid encoding HA may be synthesized by reverse
30 transcription and polymerase chain reaction (PCR) using HA RNA. As an example,

the RNA may be isolated from human influenza A/New Caledonia/20/99 (H1N1) virus or human influenza A/Indonesia/5/05 (H5N1) virus, or other influenza viruses e.g. A/Brisbane/59/2007 (H1N1), A/Solomon Islands/3/2006 (H1N1), A/Singapore/1/57 (H2N2), A/Anhui/1/2005 (H5N1), A/Vietnam/1194/2004 (H5N1),
 5 A/Teal/Hong Kong/W312/97 (H6N1), A/Hong Kong/1073/99 (H9N2), A/Brisbane/10/2007 (H3N2), A/Wisconsin/67/2005 (H3N2), A/Equine/Prague/56 (H7N7), B/Malaysia/2506/2004, B/Florida/4/2006, or from cells infected with an influenza virus. For reverse transcription and PCR, oligonucleotide primers specific for HA RNA, for example but not limited to, human influenza A/New
 10 Caledonia/20/99 (H1N1) virus HA sequences or human influenza A/Indonesia/5/05 (H5N1) virus HA0 sequences, or HA sequences from influenza subtypes A/Brisbane/59/2007 (H1N1), A/Solomon Islands/3/2006 (H1N1), A/Singapore/1/57 (H2N2), A/Anhui/1/2005 (H5N1), A/Vietnam/1194/2004 (H5N1), A/Teal/Hong Kong/W312/97 (H6N1), A/Hong Kong/1073/99 (H9N2), A/Brisbane/10/2007
 15 (H3N2), A/Wisconsin/67/2005 (H3N2), A/Equine/Prague/56 (H7N7), B/Malaysia/2506/2004, B/Florida/4/2006 may be used. Additionally, a nucleic acid encoding HA may be chemically synthesized using methods as would known to one of skill in the art.

[00171] The resulting cDNA copies of these genes may be cloned in a suitable
 20 expression vector as required by the host expression system. Examples of appropriate expression vectors for plants are described below, alternatively, baculovirus expression vector, for example, pFastBacl (InVitrogen), resulting in pFastBacl-based plasmids, using known methods, and information provided by the manufacturer's instructions may be used.

[00172] The present invention is further directed to a gene construct
 25 comprising a nucleic acid encoding 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
 30 7,125,978; which is incorporated herein by reference), or a regulatory region of Ribulose 1,5-bisphosphate carboxylase/oxygenase (RuBisCO; US 4,962,028; which is incorporated herein by reference), chlorophyll a/b binding protein (CAB; Leutwiler et

al; 1986; which is incorporated herein by reference), ST-LS1 (associated with the oxygen-evolving complex of photosystem II and described by Stockhaus et al. 1987, 1989; which is incorporated herein by reference). An example of a plastocyanin regulatory region is a sequence comprising nucleotides 10-85 of SEQ ID NO: 36, or a similar region of any one of SEQ ID NOS: 37-47.

[00173] If the construct is expressed in an insect cell, examples of regulatory elements operative in an insect cell include but are not limited to the polyhedrin promoter (Possee and Howard 1987. Nucleic Acids Research 15:10233-10248), the gp64 promoter (Kogan et al, 1995. J Virology 69:1452-1461) and the like.

10 [00174] Therefore, an aspect of the invention provides for a nucleic acid comprising a regulatory region and a sequence encoding an influenza HA. The regulatory region may be a plastocyanin regulatory element, and the influenza HA may be selected from a group of influenza strains or subtypes, comprising A/New Caledonia/20/99 (H1N1), A/Indonesia/5/05 sub-type (H5N1), A/Brisbane/59/2007 (H1N1), A/Solomon Islands/3/2006 (H1N1), A/Singapore/1/57 (H2N2),
15 A/Anhui/1/2005 (H5N1), A/Vietnam/1194/2004 (H5N1), A/Teal/Hong Kong/W312/97 (H6N1), A/Hong Kong/1073/99 (H9N2), A/Brisbane/10/2007 (H3N2), A/Wisconsin/67/2005 (H3N2), A/Equine/Prague/56 (H7N7), B/Malaysia/2506/2004, B/Florida/4/2006. Nucleic acid sequences comprising a
20 plastocyanin regulatory element and an influenza HA are exemplified herein by SEQ ID NOS: 36-47.

[00175] It is known that there may be sequence differences in the sequence of influenza hemagglutinin amino acids sequences, or the nucleic acids encoding them, when influenza virus is cultured in eggs, or mammalian cells, (e.g. MDCK cells) or
25 when isolated from an infected subject. Non-limiting examples of such differences are illustrated herein, including Example 18. Furthermore, as one of skill in the art would realize, additional variation may be observed within influenza hemagglutinins obtained from new strains as additional mutations continue to occur. Due to the known sequence variability between different influenza hemagglutinins, the present
30 invention includes VLPs that may be made using any influenza hemagglutinin provided that when expressed in a host as described herein, the influenza hemagglutinin forms a VLP.

[00176] Sequence alignments and consensus sequences may be determined using any of several software packages known in the art, for example MULTALIN (F. CORPET, 1988, Nucl. Acids Res., 16 (22), 10881-10890), or sequences may be aligned manually and similarities and differences between the sequences determined.

5 [00177] The structure of hemagglutinins is well-studied and the structures are known to be highly conserved. When hemagglutinin structures are superimposed, a high degree of structural conservation is observed (rmsd <2Å). This structural conservation is observed even though the amino acid sequence may vary in some positions (see, for example, Skehel and Wiley, 2000 Ann Rev Biochem 69:531-69; 10 Vaccaro et al 2005). Regions of hemagglutinins are also well-conserved, for example:

- Structural domains: The HA0 polypeptide is cleaved to provide mature HA. HA is a homotrimer with each monomer comprising a receptor binding domain (HA1) and a membrane-anchoring domain (HA2) linked by a single 15 disulphide bond; the N-terminal 20 residues of the HA2 subunit may also be referred to as the HA fusion domain or sequence. A 'tail' region (internal to the membrane envelope) is also present. Each hemagglutinin comprises these regions or domains. Individual regions or domains are typically conserved in length.

20  All hemagglutinins contain the same number and position of intra- and inter-molecular disulfide bridges. The quantity and position on the amino acid sequence of the cysteines that participate in disulfide bridge network is conserved among the HAs. Examples of structures illustrating the characteristic intra- and intermolecular disulfide bridges and other conserved 25 amino acids and their relative positions are described in, for example, Gamblin et al 2004 (Science 303:1838-1842). Exemplary structures and sequences include 1RVZ, 1RVX, 1RVT, 1RV0, 1RUY, 1RU7, available from the Protein Data Bank (URL: www.rcsb.org).

- Cytoplasmic tail – the majority of hemagglutinins comprise 3 cysteines at conserved positions. One or more of these cysteines may be palmitoylated as a post-translational modification.

5 [00178] Amino acid variation is tolerated in hemagglutinins of influenza viruses. This variation provides for new strains that are continually 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, or a nucleic acid
10 encoding a hemagglutinin amino acid sequence, that forms VLPs in a plant, and includes known sequences and variant sequences that may develop.

[00179] Figure 65 illustrates an example of such known variation. This figure shows a consensus amino acid sequence (SEQ ID NO: 74) for HA of the following H1N1 strains:

15 A/New Caledonia/20/99 (H1N1) (encoded by SEQ ID NO: 33),
A/Brisbane/59/2007 (H1N1) (SEQ ID NO: 48),
A/Solomon Islands/3/2006 (H1N1) (SEQ ID NO: 49) and

SEQ ID NO: 9. X1 (position 3) is A or V; X2 (position 52) is D or N; X3 (position 90) is K or R; X4 (position 99) is K or T; X5 (position 111) is Y or H; X6
20 (position 145) is V or T; X7 (position 154) is E or K; X8 (position 161) is R or K; X9 (position 181) is V or A; X10 (position 203) is D or N; X11 (position 205) is R or K; X12 (position 210) is T or K; X13 (position 225) is R or K; X14 (position 268) is W or R; X15 (position 283) is T or N; X16 (position 290) is E or K; X17 (position 432) is I or L; X18 (position 489) is N or D.

25 [00180] As another example of such variation, a sequence alignment and consensus sequence for HA of A/New Caledonia/20/99 (H1N1) (encoded by SEQ ID NO: 33), A/Brisbane/59/2007 (H1N1) (SEQ ID NO: 48), A/Solomon Islands/3/2006 (H1N1) (SEQ ID NO: 49), A/PuertoRico/8/34 (H1N1) and SEQ ID NO: 9 is shown below in Table 3.

Table 3: Sequence alignment and consensus sequence for HA of selected H1N1 strains

SEQ ID NO.		Sequence					
5	1	50					
	75	MKAKLLVLLC	TFTATYADTI	CIGYHANNST	DTVDTVLEKN	VTVTHSVNLL	
	9	MKAKLLVLLC	TFTATYADTI	CIGYHANNST	DTVDTVLEKN	VTVTHSVNLL	
	48	MKVKLLVLLC	TFTATYADTI	CIGYHANNST	DTVDTVLEKN	VTVTHSVNLL	
	49	MKVKLLVLLC	TFTATYADTI	CIGYHANNST	DTVDTVLEKN	VTVTHSVNLL	
10	76						
	Consensus	mkxkllvllc	tftatyadti	cigyhannst	dtvdtvlekn	vtvthsvnll	
15	51	100					
	75	EDSHNGKLCL	LKGIAPLQLG	NCSVAGWILG	NPECELLISK	ESWSYIVETP	
	9	EDSHNGKLCL	LKGIAPLQLG	NCSVAGWILG	NPECELLISK	ESWSYIVETP	
	48	ENSHNGKLCL	LKGIAPLQLG	NCSVAGWILG	NPECELLISK	ESWSYIVEKP	
	49	EDSHNGKLCL	LKGIAPLQLG	NCSVAGWILG	NPECELLISR	ESWSYIVEKP	
20	76						
	Consensus	exshngklcl	lkgiaplqlg	ncsvagwilg	npecellis.	eswsyive.p	
25	101	150					
	75	NPENGTCYPG	YFADYEELRE	QLSSVSSFER	FEIFPKESSW	PNHTVTGVSA	
	9	NPENGTCYPG	YFADYEELRE	QLSSVSSFER	FEIFPKESSW	PNHTVTGVSA	
	48	NPENGTCYPG	HFADYEELRE	QLSSVSSFER	FEIFPKESSW	PNHTVTGVSA	
	49	NPENGTCYPG	HFADYEELRE	QLSSVSSFER	FEIFPKESSW	PNHTTTGVSA	
30	76						
	Consensus	npengtcypg	xfadyeelre	qlssvssfer	feifpkessw	pnhtxtgvs	
35	151	200					
	75	SCSHNGKSSF	YRNLLWLTGK	NGLYPNLSKS	YVNNKEKEVL	VLWGVHHPPN	
	9	SCSHNGKSSF	YRNLLWLTGK	NGLYPNLSKS	YVNNKEKEVL	VLWGVHHPPN	
	48	SCSHNGESSF	YRNLLWLTGK	NGLYPNLSKS	YANNKEKEVL	VLWGVHHPPN	
	49	SCSHNGESSF	YKNLLWLTGK	NGLYPNLSKS	YANNKEKEVL	VLWGVHHPPN	
40	76						
	Consensus	scshngxssf	yxnlwltgk	nglypnlsks	yxnnkekevl	vlwgvhhppn	
45	201	250					
	75	IGNQRALYHT	ENAYVSVVSS	HYSRRFTPEI	AKRPKVRDQE	GRINYYWTLL	
	9	IGNQRALYHT	ENAYVSVVSS	HYSRRFTPEI	AKRPKVRDQE	GRINYYWTLL	
	48	IGDQKALYHT	ENAYVSVVSS	HYSRKFTPEI	AKRPKVRDQE	GRINYYWTLL	
	49	IGDQKALYHK	ENAYVSVVSS	HYSRKFTPEI	AKRPKVRDQE	GRINYYWTLL	
50	76		MSLLT	EVETVLSII	PSGPLKAEIA	QRLEDVFAGK	
	Consensus	igxqxalyhx	enayvsvvss	hysrxftpei	akrPkvr#qe	gRi#yywtll	
55	251	300					
	75	EPGDTIIFEA	NGNLIAPWYA	FALSRGFGSG	IITSNAPMDE	CDAKCQTPQG	
	9	EPGDTIIFEA	NGNLIAPWYA	FALSRGFGSG	IITSNAPMDE	CDAKCQTPQG	
	48	EPGDTIIFEA	NGNLIAPRYA	FALSRGFGSG	IINSNAPMDK	CDAKCQTPQG	
	49	EPGDTIIFEA	NGNLIAPRYA	FALSRGFGSG	IINSNAPMDE	CDAKCQTPQG	
50	76	NTDLEVLMEW	...LKTRPIL	SPLTKGILGF	VFTLTVP SER	GLQRRRFVQN	
	Consensus	#pgdt!ifEa	ngnLiapxya	faLsrGfgsg	!itsnaPm#x	cdakcqtPQg	
55	301	350					
	75	AINSSLPFQN	VHPVTIGEC	KYVRS AKLRM	VT.GLRNIPS	IQSRGLFGAI	
	9	AINSSLPFQN	VHPVTIGEC	KYVRS AKLRM	VT.GLRNIPS	IQSRGLFGAI	
	48	AINSSLPFQN	VHPVTIGEC	KYVRS AKLRM	VT.GLRNIPS	IQSRGLFGAI	
55	49	AINSSLPFQN	VHPVTIGEC	KYVRS AKLRM	VT.GLRNIPS	IQSRGLFGAI	

	76	ALNG.....N	GDPNNMDKAV	KLYRKLKREI	TFHGAKEISL	SYSAGALASC
	Consensus	AiNsslpfqN	vhPvtigecp	KyvRsaKlrm	vtxGlr#Ips	iqSrGlfgai
		351				400
5	75	AGFIEGGWTG	MVDGWYGYHH	QNEQSGSYAA	DQKSTQNAIN	GITNKVNSVI
	9	AGFIEGGWTG	MVDGWYGYHH	QNEQSGSYAA	DQKSTQNAIN	GITNKVNSVI
	48	AGFIEGGWTG	MVDGWYGYHH	QNEQSGSYAA	DQKSTQNAIN	GITNKVNSVI
	49	AGFIEGGWTG	MVDGWYGYHH	QNEQSGSYAA	DQKSTQNAIN	GITNKVNSVI
	76	MGLIYNRM.G	AVTTEVAFGL	VCATCEQIAD	SQHRSHRQMV	TTTNPLIRHE
10	Consensus	aGfIeggwtG	mVdgyyg%hh	qneqsgsyAa	dQkstqnain	giTNkvnsvi
		401				450
	75	EKMNTQFTAV	GKEFNKLERR	MENLNKKVDD	GFLDIWTYNA	ELLVLENER
	9	EKMNTQFTAV	GKEFNKLERR	MENLNKKVDD	GFLDIWTYNA	ELLVLENER
15	48	EKMNTQFTAV	GKEFNKLERR	MENLNKKVDD	GFIDIWTYNA	ELLVLENER
	49	EKMNTQFTAV	GKEFNKLERR	MENLNKKVDD	GFIDIWTYNA	ELLVLENER
	76	NRMVLAStTA	.KAMEQMAGS	SEQAAEAMEV	A.....S	QARQMVQAMR
	Consensus	#kMntqfTav	gKef#k\$err	mE#lnkkv#d	gfdiwtyna	#llv\$l#neR
		451				500
	75	TLDFHDSNVK	NLYEKVKSQ	KNNAKEIGNG	CFEFYHKCNN	ECMESVKNGT
	9	TLDFHDSNVK	NLYEKVKSQ	KNNAKEIGNG	CFEFYHKCNN	ECMESVKNGT
	48	TLDFHDSNVK	NLYEKVKSQ	KNNAKEIGNG	CFEFYHKCND	ECMESVKNGT
	49	TLDFHDSNVK	NLYEKVKSQ	KNNAKEIGNG	CFEFYHKCND	ECMESVKNGT
25	76	TIGTHPSSSA	GLKNDLLENL	QAYQKRMGVQ	MQRFK.....	
	Consensus	TldfHdSnvk	nLy#kvks#L	knnaKeiGng	cfeFyhkcncx	ecmesvkngt
		501				550
	75	YDYPKYSEES	KLNREKIDGV	KLESMGVYQI	LAIYSTVASS	LVLLVSLGAI
30	9	YDYPKYSEES	KLNREKIDGV	KLESMGVYQI	LAIYSTVASS	LVLLVSLGAI
	48	YDYPKYSEES	KLNREKIDGV	KLESMGVYQI	LAIYSTVASS	LVLLVSLGAI
	49	YDYPKYSEES	KLNREKIDGV	KLESMGVYQI	LAIYSTVASS	LVLLVSLGAI
	76					
	Consensus	ydyppysees	klNrekidgv	klesmgvyqi	laiystvass	lvllvslgai
35						
		551		566		
	75	SFWMCSNGSL	QCRICI			
	9	SFWMCSNGSL	QCRICI			
	48	SFWMCSNGSL	QCRICI			
40	49	SFWMCSNGSL	QCRICI			
	76					
	Consensus	sfwmcsgsl	qcrici			

The consensus sequence indicates in upper case letters amino acids common to all sequences at a designated position; lower case letters indicate amino acids common to at least half, or a majority of the sequences; the symbol ! is any one of I or V; the symbol \$ is any one of L or M; the symbol % is any one of F or Y; the symbol # is any one of N, D, Q, E, B or Z; the symbol "." is no amino acid (e.g. a deletion); X at position 3 is any one of A or V; X at position 52 is any one of E or N; X at position 90 is K or R; X at position 99 is T or K; X at position 111 is any one of Y or H; X at position 145 is any one of V or T; X at position 157 is K or E; X at position 162 is R or K; X at position 182 is V or A; X at position 203 is N or D; X at position 205 is R

or K; X at position 210 is T or K; X at position 225 is K or Y; X at position 333 is H or a deletion; X at position 433 is I or L; X at position 49) is N or D.

[00181] As another example of such variation, a sequence alignment and consensus sequence for HA of A/Anhui/1/2005 (H5N1) (SEQ ID NO: 55),
 5 A/Vietnam/1194/2004 (H5N1) and A/Indonesia/5/2006 (H5N1) (SEQ ID NO: 10) is shown below in Table 4.

Table 4: Sequence alignment and consensus sequence for HA of selected H1N1 strains

SEQ ID NO.	Sequence					
10	1	50				
	10	MEKIVLLLLAI	VSLVKSDQIC	IGYHANNSTE	QVDTIMEKNV	TVTHAQDILE
	56	MEKIVLLLFAI	VSLVKSDQIC	IGYHANNSTE	QVDTIMEKNV	TVTHAQDILE
	55	MEKIVLLLLAI	VSLVKSDQIC	IGYHANNSTE	QVDTIMEKNV	TVTHAQDILE
	Consensus	MEKIVLLlAI	VSLVKSDQIC	IGYHANNSTE	QVDTIMEKNV	TVTHAQDILE
15	51	100				
	10	KTHNGKLCDL	DGVKPLILRD	CSVAGWLLGN	PMCDEFINVP	EWSYIVEKAN
	56	KTHNGKLCDL	DGVKPLILRD	CSVAGWLLGN	PMCDEFINVP	EWSYIVEKAN
	55	KTHNGKLCDL	DGVKPLILRD	CSVAGWLLGN	PMCDEFINVP	EWSYIVEKAN
	Consensus	KTHNGKLCDL	DGVKPLILRD	CSVAGWLLGN	PMCDEFINVP	EWSYIVEKAN
20	101	150				
	10	PTNDLCYPGS	FNDYEELKHL	LSRINHFEDI	QIIPKSSWSD	HEASSGVSSA
	56	PVNDLCYPGD	FNDYEELKHL	LSRINHFEDI	QIIPKSSWSS	HEASLGVSAA
	55	PANDLCYPGN	FNDYEELKHL	LSRINHFEDI	QIIPKSSWSD	HEASSGVSSA
	Consensus	PxNDLCYPGx	FNDYEELKHL	LSRINHFEDI	QIIPKSSWSd	HEASsGVSSA
25	151	200				
	10	CPYLGSPSFF	RNVVWLIKKN	STYPTIKKSY	NNTNQEDLLV	LWGIHHPNDA
	56	CPYQGKSSFF	RNVVWLIKKN	STYPTIKRSY	NNTNQEDLLV	LWGIHHPNDA
	55	CPYQGTSPFF	RNVVWLIKKN	NTYPTIKRSY	NNTNQEDLLI	LWGIHHSNDA
	Consensus	CPYqGxpSFF	RNVVWLIKKN	stYPTIKrSY	NNTNQEDLL!	LWGIHHpNDA
30	201	250				
	10	AEQTRLYQNP	TTYISIGTST	LNQRLVPKIA	TRSKVNGQSG	RMEFFWTILK
	56	AEQTKLYQNP	TTYISVGTST	LNQRLVPRIA	TRSKVNGQSG	RMEFFWTILK
	55	AEQTKLYQNP	TTYISVGTST	LNQRLVPKIA	TRSKVNGQSG	RMDFFWTILK
	Consensus	AEQTkLYQNP	TTYIS!GTST	LNQRLVPkIA	TRSKVNGQSG	RM#FFWTILK
35	251	300				
	10	PNDAINFESN	GNFIAPEYAY	KIVKKGDSAI	MKSELEYGNC	NTKCQTPMGA
	56	PNDAINFESN	GNFIAPEYAY	KIVKKGDSTI	MKSELEYGNC	NTKCQTPMGA
	55	PNDAINFESN	GNFIAPEYAY	KIVKKGDSAI	VKSEVEYGNC	NTKCQTPIGA
	Consensus	PNDAINFESN	GNFIAPEYAY	KIVKKGDSaI	mKSe1EYGNC	NTKCQTPmGA
40	251	350				
	10	INSSMPFHNI	HPLTIGECPK	YVKSNNRLVLA	TGLRNSPQRE	SRRKKRGLFG
	56	INSSMPFHNI	HPLTIGECPK	YVKSNNRLVLA	TGLRNSPQRE	RRRKKRGLFG
	55	INSSMPFHNI	HPLTIGECPK	YVKSNNRLVLA	TGLRNSPLRE	RRRK.RGLFG
	Consensus	INSSMPFHNI	HPLTIGECPK	YVKSNNRLVLA	TGLRNSPLRE	RRRK.RGLFG

	Consensus	INSSMPFHNI	HPLTIGECPK	YVKSNrLVLA	TGLRNSPqRE	rRRKkRGLFG	
		351				400	
5	10	AIAGFIEGGW	QGMVDGWYGY	HHSNEQGSgy	AADKESTQKA	IDGVTNKVNS	
	56	AIAGFIEGGW	QGMVDGWYGY	HHSNEQGSgy	AADKESTQKA	IDGVTNKVNS	
	55	AIAGFIEGGW	QGMVDGWYGY	HHSNEQGSgy	AADKESTQKA	IDGVTNKVNS	
	Consensus	AIAGFIEGGW	QGMVDGWYGY	HHSNEQGSgy	AADKESTQKA	IDGVTNKVNS	
		401				450	
10	10	IIDKMNTQFE	AVGREFNnLE	RRIENLNKKM	EDGFLDVWty	NAELLVLMEN	
	56	IIDKMNTQFE	AVGREFNnLE	RRIENLNKKM	EDGFLDVWty	NAELLVLMEN	
	55	IIDKMNTQFE	AVGREFNnLE	RRIENLNKKM	EDGFLDVWty	NAELLVLMEN	
	Consensus	IIDKMNTQFE	AVGREFNnLE	RRIENLNKKM	EDGFLDVWty	NAELLVLMEN	
15		451				500	
	10	ERTLDFHDSN	VKNLYDKVRL	QLRDNAKELG	NGCFEFYHKC	DNECMESIRN	
	56	ERTLDFHDSN	VKNLYDKVRL	QLRDNAKELG	NGCFEFYHKC	DNECMESVRN	
	55	ERTLDFHDSN	VKNLYDKVRL	QLRDNAKELG	NGCFEFYHKC	DNECMESVRN	
	Consensus	ERTLDFHDSN	VKNLYDKVRL	QLRDNAKELG	NGCFEFYHKC	DNECMES!RN	
20		501				550	
	10	GTYNYPQYSE	EARLKREEIS	GVKLESIGTY	QILSIYSTVA	SSLALAIMMA	
	56	GTYDYPQYSE	EARLKREEIS	GVKLESIGIY	QILSIYSTVA	SSLALAIMVA	
	55	GTYDYPQYSE	EARLKREEIS	GVKLESIGTY	QILSIYSTVA	SSLALAIMVA	
25	Consensus	GTY#YPQYSE	EARLKREEIS	GVKLESIGtY	QILSIYSTVA	SSLALAIMvA	
		551		568			
	10	GLSLWMCSNG	SLQCRICI				
	56	GLSLWMCSNG	SLQCRICI				
30	55	GLSLWMCSNG	SLQCRICI				
	Consensus	GLSLWMCSNG	SLQCRICI				

The consensus sequence indicates in upper case letters amino acids common to all sequences at a designated position; lower case letters indicate amino acids common to at least half, or a majority of the sequences; the symbol ! is any one of I or V; the symbol \$ is any one of L or M; the symbol % is any one of F or Y; the symbol # is any one of N, D, Q, E, B or Z; X at position 102 is any of T, V or A; X t position 110 is any of S, D or N; X at position 156 is any of S, K or T.

[00182] The above-illustrated and described alignments and consensus sequences are non-limiting examples of variants in hemagglutinin amino acid sequences that may be used in various embodiments of the invention for the production of VLPs in a plant.

[00183] A nucleic acid encoding an amino acid sequence may be easily determined, as the codons for each amino acid are known in the art. Provision of an amino acid sequence, therefore, teaches the degenerate nucleic acid sequences that encode it. The present invention, therefore, provides for a nucleic acid sequence

encoding the hemagglutinin of those influenza strains and subtypes disclosed herein (e.g. A/New Caledonia/20/99 (H1N1)A/Indonesia/5/2006 (H5N1), A/chicken/New York/1995, A/herring gull/DE/677/88 (H2N8), A/Texas/32/2003, A/mallard/MN/33/00, A/duck/Shanghai/1/2000, A/northern pintail/TX/828189/02, A/Turkey/Ontario/6118/68(H8N4), A/shoveler/Iran/G54/03, A/chicken/Germany/N/1949(H10N7), A/duck/England/56(H11N6), A/duck/Alberta/60/76(H12N5), A/Gull/Maryland/704/77(H13N6), A/Mallard/Gurjev/263/82, A/duck/Australia/341/83 (H15N8), A/black-headed gull/Sweden/5/99(H16N3), B/Lee/40, C/Johannesburg/66, A/PuertoRico/8/34 (H1N1), A/Brisbane/59/2007 (H1N1), A/Solomon Islands 3/2006 (H1N1), A/Brisbane 10/2007 (H3N2), A/Wisconsin/67/2005 (H3N2), B/Malaysia/2506/2004, B/Florida/4/2006, A/Singapore/1/57 (H2N2), A/Anhui/1/2005 (H5N1), A/Vietnam/1194/2004 (H5N1), A/Teal/HongKong/W312/97 (H6N1), A/Equine/Prague/56 (H7N7), A/HongKong/1073/99 (H9N2)), as well as the degerenate sequences that encode the above hemagglutinins.

[00184] Further, an amino acid sequence encoded by a nucleic acid may be easily determined, as the codon or codonss for each amino acid are known. Provision of a nucleic acid, therefore, teaches an amino acid sequence encoded by it. The invention, therefore, provides for amino acid sequences of the hemagglutinin of those influenza strains and subtypes disclosed herein those disclosed herein (e.g. A/New Caledonia/20/99 (H1N1)A/Indonesia/5/2006 (H5N1), A/chicken/New York/1995, A/herring gull/DE/677/88 (H2N8), A/Texas/32/2003, A/mallard/MN/33/00, A/duck/Shanghai/1/2000, A/northern pintail/TX/828189/02, A/Turkey/Ontario/6118/68(H8N4), A/shoveler/Iran/G54/03, A/chicken/Germany/N/1949(H10N7), A/duck/England/56(H11N6), A/duck/Alberta/60/76(H12N5), A/Gull/Maryland/704/77(H13N6), A/Mallard/Gurjev/263/82, A/duck/Australia/341/83 (H15N8), A/black-headed gull/Sweden/5/99(H16N3), B/Lee/40, C/Johannesburg/66, A/PuertoRico/8/34 (H1N1), A/Brisbane/59/2007 (H1N1), A/Solomon Islands 3/2006 (H1N1), A/Brisbane 10/2007 (H3N2), A/Wisconsin/67/2005 (H3N2), B/Malaysia/2506/2004, B/Florida/4/2006, A/Singapore/1/57 (H2N2), A/Anhui/1/2005 (H5N1), A/Vietnam/1194/2004 (H5N1), A/Teal/HongKong/W312/97 (H6N1), A/Equine/Prague/56 (H7N7), A/HongKong/1073/99 (H9N2)).

[00185] In plants, influenza VLPs bud from the plasma membrane (see Example 5, and Figure 19) therefore the lipid composition of the VLPs reflects their origin. The VLPs produced according to the present invention comprise HA of one or more than one type or subtype of influenza, complexed with plant derived lipids.

5 Plant lipids can stimulate specific immune cells and enhance the immune response induced. Plant membranes are made of lipids, phosphatidylcholine (PC) and phosphatidylethanolamine (PE), and also contain glycosphingolipids, saponins, and phytosterols. Additionally, lipid rafts are also found in plant plasma membranes - these microdomains are enriched in sphingolipids and sterols. In plants, a variety of

10 phytosterols are known to occur, including stigmasterol, sitosterol, 24-methylcholesterol and cholesterol (Mongrand et al., 2004).

[00186] 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

15 thymus and liver (Tsuji M., 2006). CD1 molecules are structurally similar to major histocompatibility complex (MHC) molecules of class I and their role is to present glycolipid antigens to NKT cells (Natural Killer T cells). Upon activation, NKT cells activate innate immune cells such as NK cells and dendritic cells and also activate adaptive immune cells like the antibody-producing B cells and T-cells.

20 [00187] A variety of phytosterols may be found in a plasma membrane – the specific complement may vary depending on the species, growth conditions, nutrient resources or pathogen state, to name a few factors. Generally, beta-sitosterol is the most abundant phytosterol.

[00188] The phytosterols present in an influenza VLP complexed with a lipid

25 bilayer, such as an plasma-membrane derived envelope may provide for an advantageous vaccine composition. Without wishing to be bound by theory, plant-made VLPs complexed with a lipid bilayer, such as a plasma-membrane derived envelope, may induce a stronger immune reaction than VLPs made in other expression systems, and may be similar to the immune reaction induced by live or

30 attenuated whole virus vaccines.

[00189] Therefore, in some embodiments, the invention provides for a VLP complexed with a plant-derived lipid bilayer. In some embodiments the plant-derived lipid bilayer may comprise the envelope of the VLP.

5 [00190] The VLP produced within a plant may induce an HA comprising plant-specific N-glycans. Therefore, this invention also provides for a VLP comprising HA having plant specific N-glycans.

[00191] Furthermore, modification of N-glycan in plants is known (see for example U.S. 60/944,344; which is incorporated herein by reference) and HA having modified N-glycans may be produced. HA comprising a modified glycosylation pattern, for example with reduced fucosylated, xylosylated, or both, fucosylated and xylosylated, N-glycans may be obtained, or HA having a modified glycosylation 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 HA when compared to a wild-type plant expressing HA.

10
15

[00192] For example, which is not to be considered limiting, the synthesis of 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 (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-acetylglucosaminyltrasnferase III (GnT-III), for example but not limited to mammalian GnT-III or human GnT-III, GnT-III from other sources may also be used. Additionally, a GNT1-GnT-III hybrid enzyme, comprising the CTS of GNT1 fused to GnT-III may also be used .

20
25

30 [00193] Therefore the present invention also includes VLP's comprising HA having modified N-glycans.

[00194] Without wishing to be bound by theory, the presence of plant N-glycans on 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 by Saint-jore-Dupas et al. (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.

[00195] 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 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 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 repressors, upstream activating sequences, and mRNA instability determinants. Several of these latter elements may be located proximal to the coding region.

[00196] 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.

5 [00197] 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
10 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
15 (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 (Figure 1b or SEQ ID NO:23); US 7,125,978, which is incorporated herein by reference).

[00198] An inducible regulatory region is one that is capable of directly or
20 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,
25 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
30 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).

[00199] 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.

[00200] By "operatively linked" it is meant that the particular sequences, for example a regulatory element and a coding region of interest, interact either directly or indirectly to carry out an intended function, such as mediation or modulation of gene expression. The interaction of operatively linked sequences may, for example, be mediated by proteins that interact with the operatively linked sequences.

[00201] 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., alfalfa, potato, ginseng, pea, oat, rice, soybean, wheat, barley, sunflower, cotton and the like.

5 [00202] 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 polyadenylation signal is usually characterized by effecting the
10 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. One or more of the chimeric genetic constructs of the present invention can also include further enhancers, either translation or transcription enhancers, as may be required. These
15 enhancer regions are well known to persons skilled in the art, and can include the ATG initiation codon and adjacent sequences. The initiation codon must be in phase with the reading frame of the coding sequence to ensure translation of the entire sequence.

[00203] Non-limiting examples of suitable 3' regions are the 3' transcribed
20 non-translated regions containing a polyadenylation signal of *Agrobacterium* tumor inducing (Ti) plasmid genes, such as the nopaline synthase (Nos gene) and plant genes such as the soybean storage protein genes, the small subunit of the ribulose-1, 5-bisphosphate carboxylase (ssRUBISCO; US 4,962,028; which is incorporated herein by reference) gene, the promoter used in regulating plastocyanin expression
25 (Pwee and Gray 1993; which is incorporated herein by reference). An example of a plastocyanin promoter is described in US 7,125,978 (which is incorporated herein by reference)

[00204] As described herein, promoters comprising enhancer sequences with demonstrated efficiency in leaf expression, have been found to be effective in
30 transient expression. Without wishing to be bound by theory, attachment of upstream regulatory elements of a photosynthetic gene by attachment to the nuclear matrix may

mediate strong expression. For example up to -784 from the translation start site of the pea plastocyanin gene may be used mediate strong reporter gene expression.

[00205] To aid in identification of transformed plant cells, the constructs of this invention may be further manipulated to include plant selectable markers. Useful
5 selectable markers include enzymes that provide for resistance to chemicals such as an antibiotic for example, gentamycin, hygromycin, kanamycin, or herbicides such as phosphinothrycin, glyphosate, chlorosulfuron, and the like. Similarly, enzymes providing for production of a compound identifiable by colour change such as GUS (beta-glucuronidase), or luminescence, such as luciferase or GFP, may be used.

10 [00206] Also considered part of this invention are transgenic plants, plant cells or seeds 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
15 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
20 using tissue cultures.

[00207] 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 HAO for VLP production, in accordance with the present invention.

25 [00208] 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,
30 *Brassica* spp, soybean, bean, pea, alfalfa, potato, tomato, ginseng, and *Arabidopsis*.

[00209] 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.

[00210] By "transformation" it is meant the stable 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.

[00211] By the term "plant matter", it is meant any material derived from a plant. Plant matter may comprise an entire plant, tissue, cells, or any fraction thereof. Further, plant matter may comprise intracellular plant components, extracellular plant components, liquid or solid extracts of plants, or a combination thereof. Further, plant matter may comprise plants, plant cells, tissue, a liquid extract, or a combination thereof, from plant leaves, stems, fruit, roots or a combination thereof. Plant matter may comprise a plant or portion thereof which has not been subjected to any processing steps. However, it is also contemplated that the plant material may be subjected to minimal processing steps as defined below, or more rigorous processing, including partial or substantial protein purification using techniques commonly known within the art including, but not limited to chromatography, electrophoresis and the like.

[00212] By the term "minimal processing" it is meant plant matter, for example, a plant or portion thereof comprising a protein of interest which is partially purified to yield a plant extract, homogenate, fraction of plant homogenate or the like (i.e. minimally processed). Partial purification may comprise, but is not limited to disrupting plant cellular structures thereby creating a composition comprising soluble plant components, and insoluble plant components which may be separated for example, but not limited to, by centrifugation, filtration or a combination thereof. In this regard, proteins secreted within the extracellular space of leaf or other tissues could be readily obtained using vacuum or centrifugal extraction, or tissues could be extracted under pressure by passage through rollers or grinding or the like to squeeze or liberate the protein free from within the extracellular space. Minimal processing

could also involve preparation of crude extracts of soluble proteins, since these preparations would have negligible contamination from secondary plant products. Further, minimal processing may involve aqueous extraction of soluble protein from leaves, followed by precipitation with any suitable salt. Other methods may include large scale maceration and juice extraction in order to permit the direct use of the extract.

[00213] The plant matter, in the form of plant material or tissue may be orally delivered to a subject. The plant matter may be administered as part of a dietary supplement, along with other foods, or encapsulated. The plant matter or tissue may also be concentrated to improve or increase palatability, or provided along with other materials, ingredients, or pharmaceutical excipients, as required.

[00214] 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, 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.

[00215] It is contemplated that a plant comprising the protein of interest, or expressing the VLP comprising the protein of interest may be administered to a subject or target organism, in a variety of ways depending upon the need and the situation. For example, the protein of interest obtained from the plant may be extracted prior to its use in either a crude, partially purified, or purified form. If the protein is to be purified, then it may be produced in either edible or non-edible plants. Furthermore, if the protein is orally administered, the plant tissue may be harvested and directly feed to the subject, or the harvested tissue may be dried prior to feeding, or an animal may be permitted to graze on the plant with no prior harvest taking place. It is also considered within the scope of this invention for the harvested plant tissues to be provided as a food supplement within animal feed. If the plant tissue is being feed to an animal with little or not further processing it is preferred that the plant tissue being administered is edible.

[00216] 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 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 tristexa virus (CTV-p23), p24 of Grapevine leafroll-associated virus-2, (GLRaV-2 p24), p10 of Grapevine virus A, (GVA-p10), p14 of Grapevine virus B (GVB-p14), p10 of Heracleum latent virus (HLV-p10), or p16 of Garlic common latent virus (GCLV-p16). Therefore, a suppressor of silencing, for example, but not limited to, HcPro, TEV -p1/HC-Pro, BYV-p21, TBSV p19, TCV-CP, CMV-2b, PVX-p25, PVM-p11, PVS-p11, BScV-p16, CTV-p23, GLRaV-2 p24, GBV-p14, HLV-p10, GCLV-p16 or GVA-p10, may be co-expressed along with the nucleic acid sequence encoding the protein of interest to further ensure high levels of protein production within a plant.

[00217] Furthermore, VLPs may be produced that comprise a combination of HA subtypes. For example, VLPs may comprise one or more than one HA from the subtype H1, H2, H3, H4, H5, H6, H7, H8, H9, H10, H11, H12, H13, H14, H15, H16, or a combination thereof. Selection of the combination of HAs may be determined by the intended use of the vaccine prepared from the VLP. For example a vaccine for use in inoculating birds may comprise any combination of HA subtypes, while VLPs useful for inoculating humans may comprise subtypes one or more than one of subtypes H1, H2, H3, H5. However, other HA subtype combinations may be prepared depending upon the use of the VLP. In order to produce VLPs comprising combinations of HA subtypes, the desired HA subtype may be co-expressed within the same cell, for example a plant cell.

[00218] 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.

[00219] Therefore, the present invention further includes a suitable vector comprising the chimeric construct 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.

[00220] 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
5 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.

[00221] The constructs of the present invention can be introduced into plant cells using Ti plasmids, Ri plasmids, plant virus vectors, direct DNA transformation, micro-injection, electroporation, etc. For reviews of such techniques see for example
10 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
15 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
20 (1987); Howell et al. (Science 208: 1265, 1980), Horsch et al. (Science 227: 1229-1231, 1985), DeBlock et al., Plant Physiology 91: 694-701, 1989), *Methods for Plant Molecular Biology* (Weissbach and Weissbach, eds., Academic Press Inc., 1988), *Methods in Plant Molecular Biology* (Schuler and Zielinski, eds., Academic Press Inc., 1989), Liu and Lomonosoff (J. Virol Meth, 105:343-348, 2002.), U.S. Pat. Nos.
25 4,945,050; 5,036,006; and 5,100,792, U.S. patent application Ser. Nos. 08/438,666, filed May 10, 1995, and 07/951,715, filed Sep. 25, 1992, (all of which are hereby incorporated by reference).

[00222] Transient expression methods may be used to express the constructs of the present invention (see Liu and Lomonosoff, 2002, *Journal of Virological*
30 *Methods*, 105:343-348; which is incorporated herein by reference). Alternatively, a vacuum-based transient expression method, as described by Kapila et al. 1997 (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
5 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
10 is transient.

[00223] If the nucleotide sequence of interest encodes a product that is directly or indirectly toxic to the plant, then by using the method of the present invention, such toxicity may be reduced throughout the plant by selectively expressing the nucleotide sequence of interest within a desired tissue or at a desired stage of plant development.
15 In addition, the limited period of expression resulting from transient expression may reduce the effect when producing a toxic product in the plant. An inducible promoter, a tissue-specific promoter, or a cell specific promoter, may be used to selectively direct expression of the sequence of interest.

[00224] The recombinant HA VLPs of the present invention can be used in
20 conjunction with existing influenza vaccines, to supplement the vaccines, render them more efficacious, and 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,
25 Chiron, Roche, MedImmune, GlaxoSmithKline, Novartis, Sanofi-Aventis, Serono, Shire Pharmaceuticals and the like.

[00225] 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
30 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).

[00226] 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.

[00227] Administration of VLPs produced according to the present invention is described in Example 6. Administration of plant-made H5 VLP resulted in a significantly higher response when compared to administration of soluble HA (see Figures 21A and 21B).

[00228] As shown in Figures 26A and 26 B a subject administered A/Indonesia/5/05 H5 VLPs provided cross-protection to a challenge with influenza A/Turkey/582/06 (H5N1; "Turkey H5N1"). Administration of Indonesia H5 VLPs before challenge did not result in any loss of body mass. However in subject not administered H5VLPs, but challenged with Turkey H5N1, exhibited significant loss of body mass, and several subject died.

[00229] These data, therefore, demonstrate that plant-made influenza VLPs comprising the H5 hemagglutinin viral protein induce an immune response specific for pathogenic influenza strains, and that virus-like particles may bud from a plant plasma membrane.

[00230] Therefore, the present invention provides a composition comprising an effective dose of a VLP comprising an influenza virus HA protein, one or more than one plant lipid, and a pharmaceutically acceptable carrier. The influenza virus HA protein may be H5 Indonesia/5/2006. Also provided is a method of inducing immunity to an influenza virus infection in a subject. The method comprising administering the virus like particle comprising an influenza virus HA protein, one or more than one plant lipid, and a pharmaceutically acceptable carrier. The virus like particle may be administered to a subject orally, intradermally, intranasally, intramuscularly, intraperitoneally, intravenously, or subcutaneously.

[00231] 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, but are not limited to, those disclosed herein (e.g. A/New Caledonia/20/99

5 (H1N1)A/Indonesia/5/2006 (H5N1), A/chicken/New York/1995, A/herring gull/DE/677/88 (H2N8), A/Texas/32/2003, A/mallard/MN/33/00, A/duck/Shanghai/1/2000, A/northern pintail/TX/828189/02, A/Turkey/Ontario/6118/68(H8N4), A/shoveler/Iran/G54/03, A/chicken/Germany/N/1949(H10N7), A/duck/England/56(H11N6),

10 A/duck/Alberta/60/76(H12N5), A/Gull/Maryland/704/77(H13N6), A/Mallard/Gurjev/263/82, A/duck/Australia/341/83 (H15N8), A/black-headed gull/Sweden/5/99(H16N3), B/Lee/40, C/Johannesburg/66, A/PuertoRico/8/34 (H1N1), A/Brisbane/59/2007 (H1N1), A/Solomon Islands 3/2006 (H1N1), A/Brisbane 10/2007 (H3N2), A/Wisconsin/67/2005 (H3N2), B/Malaysia/2506/2004,

15 B/Florida/4/2006, A/Singapore/1/57 (H2N2), A/Anhui/1/2005 (H5N1), A/Vietnam/1194/2004 (H5N1), A/Teal/HongKong/W312/97 (H6N1), A/Equine/Prague/56 (H7N7), A/HongKong/1073/99 (H9N2)).

[00232] 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

20 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 (see URL: who.int/csr/disease/influenza/vaccine_recommendations1/en) .

25 Some or all of these strains may be employed in the combinations shown, or in other combinations, in the production of a vaccine composition.

[00233] More particularly, exemplary combinations may include VLPs from two or more strains or subtypes selected from the group comprising:

30 A/Brisbane/59/2007 (H1N1), an A/Brisbane/59/2007 (H1N1)-like virus, A/Brisbane/10/2007 (H3N2), an A/Brisbane/10/2007 (H3N2)-like virus, B/Florida/4/2006 or an B/Florida/4/2006-like virus.

[00234] Another exemplary combination may include VLPs from two or more strains or subtypes selected from the group comprising A/Indonesia/5/2005, an A/Indonesia/5/2005-like virus, A/Vietnam/1194/2004, an A/Vietnam/1194/2004-like virus, A/Anhui/1/05, an A/Anhui/1/05-like virus, A/goose/Guiyang/337/2006, A/goose/Guiyang/337/2006 – like virus, A/chicken/Shanxi/2/2006, or A/chicken/Shanxi/2/2006-like virus.

[00235] Another exemplary combination may include VLPs of A/Chicken/Italy/13474/99 (H7 type) or A/Chicken/British Columbia/04 (H7N3) strains of influenza.

[00236] Another exemplary combination may include VLPs of A/Chicken/HongKong/G9/97 or A/HongKong/1073/99. Another exemplary combination may comprise VLPs of A/Solomon Islands/3/2006. Another exemplary combination may comprise VLPs of A/Brisbane/10/2007. Another exemplary combination may comprise VLPs of A/Wisconsin/67/2005. Another exemplary combination may comprise VLPs of the B/Malaysia/2506/2004, B/Florida/4/2006 or B/Brisbane/3/2007 strains or subtypes.

[00237] 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. 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.

[00238] Therefore, the invention provides for compositions comprising VLPs of two or more strains or subtypes.

[00239] VLPs of enveloped viruses generally acquire their envelope from the membrane they bud through. Plant plasma membranes have a phytosterol complement that may have immunostimulatory effects. To investigate this possibility, plant-made H5 VLPs were administered to animals in the presence or absence of an adjuvant, and the HAI (hemagglutination inhibition antibody response) determined (Figures 22A, 22B). In the absence of an added adjuvant plant-made H5

VLPs demonstrate a significant HAI, indicative of a systemic immune response to administration of the antigen. Furthermore, the antibody isotype profiles of VLPs administered in the present or absence of adjuvant are similar (Figure 23A).

Table 5 lists sequences provided in various embodiments of the invention..

5 **Table 5: Sequence description for sequence identifiers.**

SEQ ID No	Sequence Description	In Disclosure
1	N terminal H1 fragment	Figure 5a
2	C terminal H1 fragment	Figure 5b
3	H5 coding sequence	Figure 6
4	primer Plato-443c	Figure 7a
5	primer SpHA(Ind)-Plasto.r	Figure 7b
6	primer Plasto-SpHA(Ind).c	Figure 7c
7	primer HA(Ind)-Sac.r	Figure 7d
8	Sequence of the alfalfa plastocyanin-based expression cassette used for the expression of H1	Figure 1
9	HA1 peptide sequence (A/New Caledonia/20/99)	Figure 8a
10	HA5 peptide sequence (A/Indonesia/5/2006)	Figure 8b
11	Influenza A Subtype H7 coding sequence (A/chicken/New York/1995)	Figure 9
12	Influenza A Subtype H2 coding sequence (A/herring gull/DE/677/88 (H2N8))	Figure 10a
13	Influenza A Subtype H3 coding sequence (A/Texas/32/2003)	Figure 10b
14	Influenza A Subtype H4 coding sequence (A/mallard/MN/33/00)	Figure 10c
15	Influenza A Subtype H5 coding sequence (A/duck/Shanghai/1/2000)	Figure 10d
16	Influenza A Subtype H6 coding sequence (A/northern pintail/TX/828189/02)	Figure 10e
17	Influenza A Subtype H8 coding sequence (A/Turkey/Ontario/6118/68(H8N4))	Figure 10f
18	Influenza A Subtype H9 coding sequence (A/shoveler/Iran/G54/03)	Figure 10g
19	Influenza A Subtype H10 coding sequence (A/chicken/Germany/N/1949 (H10N7))	Figure 10h
20	Influenza A Subtype H11 coding sequence (A/duck/England/56(H11N6))	Figure 10i
21	Influenza A Subtype H12 coding sequence (A/duck/Alberta/60/76(H12N5))	Figure 10j

SEQ ID No	Sequence Description	In Disclosure
22	Influenza A Subtype H13 coding sequence (A/Gull/Maryland/704/77 (H13N6))	Figure 10k
23	Influenza A Subtype H14 coding sequence (A/Mallard/Gurjev/263/82)	Figure 10l
24	Influenza A Subtype H15 coding sequence (A/duck/Australia/341/83 (H15N8))	Figure 10m
25	Influenza A Subtype H16 coding sequence (A/black-headed gull/Sweden/5/99(H16N3))	Figure 10n
26	Influenza B HA coding sequence (B/Lee/40)	Figure 10o
27	Influenza C HA coding sequence (C/Johannesburg/66)	Figure 10p
28	Complete HAO H1 sequence	Figure 5c
29	Primer XmaI-pPlas.c	Figure 10q
30	Primer SacI-ATG-pPlas.r	Figure 10r
31	Primer SacI-PlasTer.c	Figure 10s
32	Primer EcoRI-PlasTer.r	Figure 10t
33	A/New Caledonia/20/99 (H1N1) GenBank Accession No. AY289929	Figure 16
34	M. Sativa protein disulfide isomerase GenBank Accession No. Z11499	Figure 17
35	A/PuertoRico/8/34 (H1N1) GenBank Accession No. NC_002016.1	Figure 18
36	Clone 774: DNA from DraIII to SacI comprising plastocyanin regulatory region operatively linked to sequence encoding HA of A/Brisbane/59/2007 (H1N1)	Figure 28
37	Clone 775: DNA from DraIII to SacI comprising plastocyanin regulatory region operatively linked to sequence encoding HA of A/Solomon Islands 3/2006 (H1N1)	Figure 29
38	Clone 776: DNA from DraIII to SacI comprising plastocyanin regulatory region operatively linked to sequence encoding HA of A/Brisbane 10/2007 (H3N2)	Figure 30
39	Clone 777: DNA from DraIII to SacI comprising plastocyanin regulatory region operatively linked to sequence encoding HA of A/Wisconsin/67/2005 (H3N2)	Figure 31
40	Clone 778: DNA from DraIII to SacI comprising plastocyanin regulatory region operatively linked to sequence encoding HA of B/Malaysia/2506/2004	Figure 32

SEQ ID No	Sequence Description	In Disclosure
41	Clone 779: DNA from DraIII to SacI comprising plastocyanin regulatory region operatively linked to sequence encoding HA of B/Florida/4/2006	Figure 33
42	Clone 780: DNA from DraIII to SacI comprising plastocyanin regulatory region operatively linked to sequence encoding HA of A/Singapore/1/57 (H2N2)	Figure 34
43	Clone 781: DNA from DraIII to SacI comprising plastocyanin regulatory region operatively linked to sequence encoding HA of A/Anhui/1/2005 (H5N1)	Figure 35
44	Clone 782: DNA from DraIII to SacI comprising plastocyanin regulatory region operatively linked to sequence encoding HA of A/Vietnam/1194/2004 (H5N1)	Figure 36
45	Clone 783: DNA from DraIII to SacI comprising plastocyanin regulatory region operatively linked to sequence encoding HA of A/Teal/HongKong/W312/97 (H6N1)	Figure 37
46	Clone 784: DNA from DraIII to SacI comprising plastocyanin regulatory region operatively linked to sequence encoding HA of A/Equine/Prague/56 (H7N7)	Figure 38
47	Clone 785: DNA from DraIII to SacI comprising plastocyanin regulatory region operatively linked to sequence encoding HA of A/HongKong/1073/99 (H9N2)	Figure 39
48	Clone 774 HA amino acid sequence A/Brisbane/59/2007 (H1N1)	Figure 40A
49	Clone 775 HA amino acid sequence A/Solomon Islands 3/2006 (H1N1)	Figure 40B
50	Clone 776 HA amino acid sequence A/Brisbane 10/2007 (H3N2)	Figure 41A
51	Clone 777 HA amino acid sequence A/Wisconsin/67/2005 (H3N2)	Figure 41B
52	Clone 778 HA amino acid sequence B/Malaysia/2506/2004	Figure 42A
53	Clone 779 HA amino acid sequence B/Florida/4/2006	Figure 42B
54	Clone 780 HA amino acid sequence A/Singapore/1/57 (H2N2)	Figure 43A
55	Clone 781 HA amino acid sequence A/Anhui/1/2005 (H5N1)	Figure 43B

SEQ ID No	Sequence Description	In Disclosure
56	Clone 782 HA amino acid sequence A/Vietnam/1194/2004 (H5N1)	Figure 44A
57	Clone 783 HA amino acid sequence A/Teal/HongKong/W312/97 (H6N1)	Figure 44B
58	Clone 784 HA amino acid sequence A/Equine/Prague/56 (H7N7)	Figure 45A
59	Clone 785 HA amino acid sequence A/HongKong/1073/99 (H9N2)	Figure 45B
60	HA expression cassette comprising alfalfa plastocyanin promoter and 5' UTR, hemagglutinin coding sequence of H5 from A/Indonesia/5/2005 (Construct # 660) , alfalfa plastocyanin 3' UTR and terminator sequences	Figure 51
61	HA expression cassette comprising alfalfa plastocyanin promoter and 5' UTR, hemagglutinin coding sequence of H1 from A/New Caledonia/20/1999 (Construct # 540) , alfalfa plastocyanin 3' UTR and terminator sequences	Figure 52
62	HA expression cassette comprising alfalfa plastocyanin promoter and 5' UTR, hemagglutinin coding sequence of H1 from A/Brisbane/59/2007 (construct #774) , alfalfa plastocyanin 3' UTR and terminator sequences	Figure 53
63	HA expression cassette comprising alfalfa plastocyanin promoter and 5' UTR, hemagglutinin coding sequence of H1 from A/Solomon Islands/3/2006 (H1N1) (construct #775) , alfalfa plastocyanin 3' UTR and terminator sequences	Figure 54
64	HA expression cassette comprising alfalfa plastocyanin promoter and 5' UTR, hemagglutinin coding sequence of H2 from A/Singapore/1/57 (H2N2) (construct # 780) , alfalfa plastocyanin 3' UTR and terminator sequences	Figure 55
65	HA expression cassette comprising alfalfa plastocyanin promoter and 5' UTR, hemagglutinin coding sequence of H5 from A/Anhui/1/2005 (H5N1) (Construct# 781) , alfalfa plastocyanin 3' UTR and terminator sequences	Figure 56

SEQ ID No	Sequence Description	In Disclosure
66	HA expression cassette comprising alfalfa plastocyanin promoter and 5' UTR, hemagglutinin coding sequence of H5 from A/Vietnam/1194/2004 (H5N1) (Construct # 782) , alfalfa plastocyanin 3' UTR and terminator sequences	Figure 57
67	HA expression cassette comprising alfalfa plastocyanin promoter and 5' UTR, hemagglutinin coding sequence of H6 from A/Teal/Hong Kong/W312/97 (H6N1) (Construct # 783) , alfalfa plastocyanin 3' UTR and terminator sequences	Figure 58
68	HA expression cassette comprising alfalfa plastocyanin promoter and 5' UTR, hemagglutinin coding sequence of H9 from A/Hong Kong/1073/99 (H9N2) (Construct # 785) , alfalfa plastocyanin 3' UTR and terminator sequences	Figure 59
69	HA expression cassette comprising alfalfa plastocyanin promoter and 5' UTR, hemagglutinin coding sequence of H3 from A/Brisbane/10/2007 (H3N2) , alfalfa plastocyanin 3' UTR and terminator sequences	Figure 60
70	HA expression cassette comprising alfalfa plastocyanin promoter and 5' UTR, hemagglutinin coding sequence of H3 from A/Wisconsin/67/2005 (H3N2) , alfalfa plastocyanin 3' UTR and terminator sequences	Figure 61
71	HA expression cassette comprising alfalfa plastocyanin promoter and 5' UTR, hemagglutinin coding sequence of H7 from A/Equine/Prague/56 (H7N7) , alfalfa plastocyanin 3' UTR and terminator sequences	Figure 62
72	HA expression cassette comprising alfalfa plastocyanin promoter and 5' UTR, hemagglutinin coding sequence of HA from B/Malaysia/2506/2004 , alfalfa plastocyanin 3' UTR and terminator sequences	prophetic Figure 63

SEQ ID No	Sequence Description	In Disclosure
73	HA expression cassette comprising alfalfa plastocyanin promoter and 5' UTR, hemagglutinin coding sequence of HA from B/Florida/4/2006, alfalfa plastocyanin 3' UTR and terminator sequences	Figure 64
74	Consensus of SEQ ID NO: 49, 48, 33 and 9	Figure 65
75	Amino acid sequence of H1 New Caledonia (AAP34324.1) encoded by SEQ ID NO: 33	Figure 67
76	Amino acid sequence of H1 Puerto Rico (NC_0409878.1) encoded by SEQ ID NO: 35	Figure 68

[00240] The invention will now be described in detail by way of reference only to the following non-limiting examples.

Methods and Materials

5 1. Assembly of expression cassettes

[00241] All manipulations were done using the general molecular biology protocols of Sambrook and Russell (2001; which is incorporated herein by reference). The first cloning step consisted in assembling a receptor plasmid containing upstream and downstream regulatory elements of the alfalfa plastocyanin gene. The

10 plastocyanin promoter and 5'UTR sequences were amplified from alfalfa genomic DNA using oligonucleotide primers **XmaI**-pPlas.c (SEQ ID NO: 29; Figure 10Q) and **SacI**-ATG-pPlas.r (SEQ ID NO: 30; Figure 10R). 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

15 pCAMBIApromo Plasto. Similarly, the 3'UTR sequences and terminator of the plastocyanin gene was amplified from alfalfa genomic DNA using the following primers: **SacI**-PlasTer.c (SEQ ID NO: 31; Figure 10S) and **EcoRI**-PlasTer.r (SEQ ID NO: 32; Figure 10T), and the product was digested with SacI and EcoRI before being inserted into the same sites of pCAMBIApromoPlasto to create

20 pCAMBIAPlasto.

[00242] 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: 33; Figure 16) lacking the signal peptide coding sequence at the 5' end and the transmembrane domain coding sequence at the 3' end. A BglII restriction site was added at the 5' end of the coding sequence 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: 1 (Figure 5A). A second fragment encoding the C-terminal end of the H1 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: 2; Figure 5B).

[00243] 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 32-103; Accession No. Z11499; SEQ ID NO: 34; Figure 17) resulting in a PDI-H1 chimeric gene downstream of the plastocyanin regulatory elements. The sequence of the plastocyanin-based cassette containing the PDI signal peptide is presented in Figure 1 (SEQ ID NO:8). The resulting plasmid contained H1 coding region fused to the PDI signal peptide and flanked by plastocyanin regulatory elements. The addition of the C-terminal end coding region (encoding the transmembrane domain and the cytoplasmic tail) was obtained by inserting the synthesized fragment (SEQ ID NO: 2; Figure 5B) previously digested with KpnI and SacI, into the H1 expression plasmid. The resulting plasmid, named 540, is presented in Figure 11 (also see Figure 2A).

2. Assembly of H5 expression cassette

[00244] A fragment encoding hemagglutinin from influenza strain A/Indonesia/5/05 (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: 3 (Figure 6). The H5 coding

region was cloned into a plastocyanin-based expression cassette by the PCR-based ligation method presented in Darveau et al. (1995). Briefly, a first PCR amplification was obtained using primers Plato-443c (SEQ ID NO: 4; Figure 7A) and SpHA(Ind)-Plasto.r (SEQ ID NO:5; Figure 7B) and pCAMBIA promoPlasto as template. In parallel, a second amplification was performed with primers Plasto-SpHA(Ind).c (SEQ ID NO: 6; Figure 7C) and HA(Ind)-Sac.r (SEQ ID NO:7; Figure 7D) with H5 coding fragment as template. The amplification obtained from both reactions were mixed together and the mixture served as template for a third reaction (assembling reaction) using Plato-443c (SEQ ID NO: 4; Figure 7A) and HA(Ind)-Sac.r (SEQ ID NO: 7; Figure 7D) 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 2B (also see Figure 11).

[00245] The cassette encoding the soluble form of H1 was prepared by replacing the region coding for the transmembrane domain and the cytoplasmic tail in 540 by a fragment encoding the leucine zipper GCN4 pII variant (Harbury et al, 1993, Science 1993; 262: 1401-1407). This fragment was synthesized with flanking KpnI and SacI sites to facilitate cloning. The plasmid resulting from this replacement was named 544 and the expression cassette is illustrated in figure 11.

[00246] A fusion between the tobacco etch virus (TEV) 5'UTR and the open reading frame of the influenza A/PR/8/34 M1 gene (Acc. # NC_002016) was synthesized with a flanking SacI site added downstream of the stop codon. The fragment was digested with SwaI (in the TEV 5'UTR) and SacI, and cloned into a 2X35S/TEV based expression cassette in a pCAMBIA binary plasmid. The resulting plasmid bore the M1 coding region under the control of a 2X35S/TEV promoter and 5'UTR and the NOS terminator (construct 750; figure 11).

[00247] 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.

3. Preparation of plant biomass, inoculum, agroinfiltration, and harvesting

- [00248] *Nicotiana benthamiana* or *Nicotiana tabacum* 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
- 10 [00249] *Agrobacteria* transfected with constructs 660, 540, 544, 750 or 35SHcPro 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
- 15 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
- 20 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.

4. Leaf sampling and total protein extraction

- 25 [00250] 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 7.4, 0.15 M NaCl, and 1 mM phenylmethanesulfonyl fluoride. After homogenization, the slurries were centrifuged at 20,000 g for 20 min at 4°C and these
- 30 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.

5. Size exclusion chromatography of protein extract

[00251] Size exclusion chromatography (SEC) columns of 32 ml Sephacryl™
5 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
10 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-
15 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.

6. Protein Analysis and Immunoblotting

[00252] Protein concentrations were determined by the BCA protein assay
20 (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).

[00253] Proteins from elution fraction from SEC were precipitated with acetone (Bollag et al., 1996), resuspended in 1/5 volume in equilibration/elution
25 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.

[00254] 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). Horseradish peroxidase–enzyme conjugation of human IgG antibody was carried out by using the EZ-Link Plus® Activated Peroxidase conjugation kit (Pierce, Rockford, IL).

Table 6: 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 0
H1	A/Solomon Islands/3/2006 (H1N1)	Reducing	NIBSC 07/104	1:2000	Rabbit anti-sheep (JIR 313-035-045)	1:10 0
H1	A/New Caledonia/20/99 (H1N1)	Reducing	FII 10-I50	4 µg/ml	Goat anti-mouse (JIR 115-035-146)	1:10 0
H2	A/Singapore/1/57 (H2N2)	Non-reducing	NIBSC 00/440	1:1000	Rabbit anti-sheep (JIR 313-035-045)	1:10 0
H5	A/Indonesia/5/2005 (H5N1)	Reducing	ITC IT-003-005V	1:4000	Goat anti-rabbit (JIR 111-035-144)	1:10 0
H5	A/Anhui/1/2005 (H5N1)	Reducing	NIBSC 07/338	1:750	Rabbit anti-sheep (JIR 313-035-045)	1:10 0
H5	A/Vietnam/1194/2004 (H5N1)	Non-reducing	ITC IT-003-005	1:2000	Goat anti-rabbit (JIR 111-035-144)	1:10 0
H6	A/Teal/Hong Kong/W312/97	Non-reducing	BEI NR 663	1:500	Rabbit anti-sheep	1:10 0

	(H6N1)				(JIR 313-035-045)	
H9	A/Hong Kong/1073/99 (H9N2)	Reducing	NIBSC 07/146	1:1000	Rabbit anti-sheep (JIR 313-035-045)	1:10 000

FII: Fitzgerald Industries International, Concord, MA, USA;

NISBIC: National Institute for Biological Standards and Control;

JIR: Jackson ImmunoResearch, West Grove, PA, USA;

BEI NR: Biodefense and emerging infections research resources repository;

ITC: Immune Technology Corporation, Woodside, NY, USA;

5

[00255] Hemagglutination assay for H5 was based on a method described by Nayak. and Reichl (2004). Briefly, serial double dilutions of the test samples (100 μ L) were made in V-bottomed 96-well microtiter plates containing 100 μ L PBS, leaving 100 μ L of diluted sample per well. One hundred microliters of a 0.25% turkey red blood cells suspension (Bio Link Inc., Syracuse, NY) were added to each well, and plates were incubated for 2h at room temperature. The reciprocal of the highest dilution showing complete hemagglutination was recorded as HA activity. In parallel, a recombinant HA standard (A/Vietnam/1203/2004 H5N1) (Protein Science Corporation, Meriden, CT) was diluted in PBS and run as a control on each plate.

10

15

7. Sucrose gradient ultracentrifugation

[00256] One milliliter of fractions 9, 10 and 11 eluted from the gel filtration chromatography on H5-containing biomass were pooled, loaded onto a 20-60% (w/v) discontinuous sucrose density gradient, and centrifuged 17,5 h at 125 000 g (4°C). The gradient was fractionated in 19 3-mL fractions starting from the top, and dialyzed to remove sucrose prior to immunological analysis and hemagglutination assays.

20

8. Electron microscopy

[00257] Elution fractions from SEC to be observed by electron microscopy (EM) were first concentrated using 30 MWCO ultrafiltration units (Millipore, Billerica, MA, USA). The concentrated fractions were fixed in PBS pH 7.4 containing 2% glutaraldehyde for 24 h at 4°C. Once fixed the samples were adsorbed onto Formvar-coated 200-mesh nickel grids (Canemco, Lakefield, Canada) for 2 min, and the grids were washed twice with deionized water before being stained in 1%

25

phosphotungstic acid. Observation was performed under transmission electron microscopy at magnifications ranging from 10,000X to 150,000X (for images in Figures 4A and 4B).

[00258] Alternately, one hundred microliters of the samples to be examined were placed in an Airfuge ultracentrifugation tube (Beckman Instruments, Palo Alto, CA, USA). A grid was placed at the bottom of the tube which was then centrifuged 5 min at 120 000 g. The grid was removed, gently dried, and placed on a drop of 3% phosphotungstic acid at pH 6 for staining. Grids were examined on a Hitachi 7100 transmission electron microscope (TEM) (for images in Figures 14B, 15B and 15C).

[00259] For images in Figure 19, Leaf blocks of approximately 1 mm³ were fixed in PBS containing 2,5% glutaraldehyde and washed in PBS containing 3% sucrose before a post-fixation step in 1,33% osmium tetroxide. Fixed samples were imbedded in Spurr resin and ultrathin layers were laid on a grid. Samples were positively stained with 5% uranyl acetate and 0,2% lead citrate before observation. Grids were examined on a Hitachi 7100 transmission electron microscope (TEM).

9. Plasma membrane lipid analysis

[00260] Plasma membranes (PM) were obtained from tobacco leaves and cultured BY2 cells after cell fractionation according to Mongrand *et al.* by partitioning in an aqueous polymer two-phase system with polyethylene glycol 3350/dextran T-500 (6.6% each). All steps were performed at 4 °C.

[00261] Lipids were extracted and purified from the different fractions according to Bligh and Dyer. Polar and neutral lipids were separated by monodimensional HP-TLC using the solvent systems described in Lefebvre *et al.*. Lipids of PM fractions were detected after staining with copper acetate as described by Macala *et al.* Lipids were identified by comparison of their migration time with those of standards (all standards were obtained from Sigma-Aldrich, St-Louis, MO, USA, except for SG which was obtained from Matreya, Pleasant Gap, PA, USA).

10. H5 VLP purification

[00262] Frozen 660-infiltrated leaves of *N. benthamiana* were homogenized in 1.5 volumes of 50 mM Tris pH 8, NaCl 150 mM and 0.04% sodium meta-bisulfite using a commercial blender. The resulting extract was supplemented with 1 mM PMSF and adjusted to pH 6 with 1 M acetic acid before being heated at 42°C for 5 min. Diatomaceous earth (DE) was added to the heat-treated extract to adsorb the contaminants precipitated by the pH shift and heat treatment, and the slurry was filtered through a Whatman paper filter. The resulting clarified extract was centrifuged at 10,000 x g for 10 minutes at RT to remove residual DE, passed through 0.8/0.2 µm Acropack 20 filters and loaded onto a fetuin-agarose affinity column (Sigma-Aldrich, St-Louis, MO, USA). Following a wash step in 400 mM NaCl, 25 mM Tris pH 6, bound proteins were eluted with 1.5 M NaCl, 50 mM MES pH 6. Eluted VLP were supplemented with Tween-80 to a final concentration of 0.0005% (v/v). VLP were concentrated on a 100 kDa MWCO Amicon membrane, centrifuged at 10,000 x g for 30 minutes at 4°C and resuspended in PBS pH 7.4 with 0.01% Tween-80 and 0.01% thimerosal. Suspended VLPs were filter-sterilized before use.

11. Animal studies

Mice

[00263] Studies on the immune response to influenza VLP administration were performed with 6-8 week old female BALB/c mice (Charles River Laboratories). Seventy mice were randomly divided into fourteen groups of five animals. Eight groups were used for intramuscular immunization and six groups were used to test intranasal route of administration. All groups were immunized in a two-dose regiment, the boost immunization being done 3 weeks following the first immunization.

[00264] For intramuscular administration in hind legs, unanaesthetized mice were immunized with either the plant-made VLP H5 vaccine (0.1, 1, 5 or 12 µg), or a control hemagglutinin (HA) antigen. The control HA comprised recombinant soluble hemagglutinin produced based on strain A/Indonesia/5/05 H5N1 and purified from 293 cell culture (Immune Technology Corp., New York, USA) (used at 5 µg per injection unless otherwise indicated). Buffer control was PBS. This antigen consists of amino acids 18-530 of the HA protein, and has a His-tag and a modified cleavage

site. Electron microscopy confirmed that this commercial product is not in the form of VLPs.

[00265] To measure the effect of adjuvant, two groups of animals were immunized with 5 µg plant-made VLP H5 vaccine plus one volume Alhydrogel 2% (alum, Accurate Chemical & Scientific Corporation, Westbury, NY, US) or with 5 µg recombinant hemagglutinin purified from 293 cell culture plus 1 volume alum. Seventy mice were randomly divided into fourteen groups of five animals. Eight groups were used for intramuscular immunization and six groups were used to test intranasal route of administration. All groups were immunized according to a prime-boost regimen, the boost immunization performed 3 weeks following the first immunization.

[00266] For intramuscular administration in hind legs, unanaesthetized mice were immunized with the plant-made H5 VLP (0.1, 1, 5 or 12 µg), or the control hemagglutinin (HA) antigen (5 µg) or PBS. All antigen preparations were mixed with Alhydrogel 1% (alum, Accurate Chemical & Scientific Corporation, Westbury, NY, US) in a 1:1 volume ratio prior to immunizations. To measure the effect of adjuvant, two groups of animals were immunized with either 5 µg plant-made VLP H5 vaccine or with 5 µg of control HA antigen without any adjuvant.

[00267] For intranasal administration, mice were briefly anaesthetized by inhalation of isoflurane using an automated induction chamber. They were then immunized by addition of 4 µl drop/nostril with the plant-made VLP vaccine (0.1 or 1 µg), or with control HA antigen (1 µg) or with PBS. All antigen preparations were mixed with chitosan glutamate 1% (Protosan, Novamatrix/ FMC BioPolymer, Norway) prior to immunizations. The mice then breathed in the solutions. To verify the effect of adjuvant with the intranasal route of administration, two groups of animals were immunized with 1 µg plant-made VLP H5 vaccine or with 1 µg control HA antigen.

Ferrets

[00268] Ten groups of 5 ferrets (male, 18-24 weeks old, mass of approx 1 kg) were used. Treatment for each group is as described in Table 7. The adjuvant used

was Alhydrogel (alum) (Superfos Biosector, Denmark) 2% (final=1%). Vaccine composition was membrane-associated A/Indonesia/5/05 (H5N1) VLPs produced as described. The vaccine control (positive control) was a fully glycosylated membrane-bound recombinant H5 from Indonesia strain produced using adenovirus in 293 cell culture by Immune Technology Corporation (ITC).

Table 7. Treatment groups

Group	n	Product injected to animals	Route of administration	Adjuvant
1	5	PBS (negative control)	i.m.*	-
2	5	Vaccine-plant, 1 µg	i.m.	-
3	5	Vaccine-plant, 1 µg	i.m.	Alum
4	5	Vaccine-plant, 5 µg	i.m.	-
5	5	Vaccine-plant, 5 µg	i.m.	Alum
6	5	Vaccine-plant, 7.5 µg	i.m.	-
7	5	Vaccine-plant, 15 µg	i.m.	-
8	5	Vaccine-plant, 15 µg	i.m.	Alum
9	5	Vaccine-plant, 30 µg	i.m.	-
10	5	Vaccine-control, 5 µg	i.m.	-

*i.m.: intramuscular

[00269] Ferrets were assessed for overall health and appearance (body weight, rectal temperature, posture, fur, movement patterns, breathing, excrement) regularly during the study. Animals were immunized by intramuscular injection (0.5-1.0 total volume) in quadriceps at day 0, 14 and 28; for protocols incorporating adjuvant, the vaccine composition was combined with Alhydrogel immediately prior to immunization in a 1:1 volume ratio). Serum samples were obtained on day 0 before immunizing, and on day 21 and 35. Animals were sacrificed (exsanguination/cardiac puncture) on days 40-45, and , spleens were collected and necropsy performed .

[00270] Anti-influenza antibody titres may be quantified in ELISA assays using homologous or heterologous inactivated H5N1 viruses.

[00271] Hemagglutination inhibitory antibody titers of serum samples (pre-immune, day 21 and day 35) were evaluated by microtiter HAI as described (Aymard et al 1973). Briefly, sera were pretreated with receptor-destroying enzyme, heat-inactivated and mixed with a suspension of erythrocytes (washed red blood cells-
5 RBC). Horse washed RBC (10%) from Lampire are recommended and considering that the assay may vary depending of the source of the RBC (horse-dependant), washed RBCs from 10 horses have been tested to select the most sensitive batch. Alternately, turkey RBC may be used. Antibody titer was expressed as the reciprocal of the highest dilution which completely inhibits hemagglutination.

10 [00272] Cross-reactive HAI titers: HAI titers of ferrets immunized with a vaccine for the A/Indonesia/5/05 (clade 2.1) were measured using inactivated H5N1 influenza strains from another subclade or clade such as the clade 1 Vietnam strains A/Vietnam/1203/2004 and A/Vietnam/1194/2004 or the A/Anhui/01/2005 (subclade 2.3) or the A/turkey/Turkey/1/05 (subclade 2.2). All analyses were performed on
15 individual samples.

[00273] Data analysis: Statistical analysis (ANOVA) will be performed on all data to establish if differences between groups are statistically significant.

Experimental design for lethal challenge(mice)

[00274] One hundred twenty eight mice were randomly divided into sixteen
20 groups of eight animals, one group being unimmunized and not challenged (negative control). All groups were immunized via intramuscular administration in a two-dose regimen, the second immunization being done 2 weeks following the first immunization.

[00275] For intramuscular administration in hind legs, unanaesthetized mice
25 were immunized with the plant-made H5 VLP (1, 5 or 15 µg), or 15 µg of control HA antigen or PBS. All antigen preparations were mixed with one volume of Alhydrogel 1% prior to immunizations (alum, Accurate Chemical & Scientific Corporation, Westbury, NY, US).

[00276] During the immunization period, mice were weighted once a week and
30 observation and monitored for local reactions at the injection site.

[00277] Twenty two days following the second immunization, anesthetized mice were challenged intranasally (i.n.) into a BL4 containment laboratory (P4-Jean Mérieux-INSERM, Lyon, France) with 4.09×10^6 50% cell culture infective dose (CCID₅₀) of influenza A/Turkey/582/06 virus (kindly provided by Dr. Bruno Lina, Lyon University, Lyon, France). Following challenge, mice were observed for ill clinical symptoms and weighed daily, over a fourteen day period. Mice with severe infection symptoms and weight loss of $\geq 25\%$ were euthanized after anaesthesia.

Blood collection, lung and nasal washes and spleen collection

[00278] Lateral saphenous vein blood collection was performed fourteen days after the first immunization and fourteen days after second immunization on unanaesthetized animal. Serum was collected by centrifuging at 8000 g for 10 min.

[00279] Four weeks after second immunisation, mice were anaesthetized with CO₂ gas and immediately upon termination, cardiac puncture was used to collect blood.

[00280] After final bleeding, a catheter was inserted into the trachea towards the lungs and one ml of cold PBS-protease inhibitor cocktail solution was put into a 1cc syringe attached to the catheter and injected into the lungs and then removed for analysis. This wash procedure was performed two times. The lung washes were centrifuged to remove cellular debris. For nasal washes, a catheter was inserted towards the nasal area and 0.5 ml of the PBS-protease inhibitor cocktail solution was pushed through the catheter into the nasal passages and then collected. The nasal washes were centrifuged to remove cellular debris. Spleen collection was performed on mice immunized intramuscularly with 5 µg of adjuvanted plant-made vaccine or 5 µg adjuvanted recombinant H5 antigen as well as on mice immunized intranasally with 1 µg of adjuvanted plant-made vaccine or 1 µg adjuvanted recombinant H5 antigen. Collected spleens were placed in RPMI supplemented with gentamycin and mashed in a 50 ml conical tube with plunger from a 10 ml syringe. Mashed spleens were rinsed 2 times and centrifuged at 2000 rpm for 5 min and resuspended in ACK lysing buffer for 5 min at room temperature. The splenocytes were washed in PBS-gentamycin, resuspended in 5% RPMI and counted. Splenocytes were used for proliferation assay.

Antibody titers

[00281] Anti-influenza antibody titers of sera were measured at 14 days after the first immunization as well as 14 and 28 days after the second immunisation. The titer were determined by enzyme-linked immunosorbent assay (ELISA) using the inactivated virus A/Indonesia/5/05 as the coating antigen. The end-point titers were expressed as the reciprocal value of the highest dilution that reached an OD value of at least 0.1 higher than that of negative control samples.

[00282] For antibody class determination (IgG1, IgG2a, IgG2b, IgG3, IgM), the titers were evaluated by ELISA as previously described.

10 *Hemagglutination inhibition (HI) titers*

[00283] Hemagglutination inhibition (HI) titers of sera were measured at 14 and 28 days after the second immunisation as previously described (WHO 2002; Kendal 1982). Inactivated virus preparations from strains A/Indonesia/5/05 or A/Vietnam/1203/2004 were used to test mouse serum samples for HI activity. Sera were pre-treated with receptor-destroying enzyme II (RDE II) (Denka Seiken Co., Tokyo, Japan) prepared from *Vibrio cholerae* (Kendal 1982). HI assays were performed with 0.5% turkey red blood cells. HI antibody titres were defined as the reciprocal of the highest dilution causing complete inhibition of agglutination.

Examples

20 **Example 1: Transient expression of influenza virus A/Indonesia/5/05 (H5N1) hemagglutinin by agroinfiltration in N. benthamiana plants**

[00284] The ability of the transient expression system to produce influenza hemagglutinin was determined through the expression of the H5 subtype from strain A/Indonesia/5/05 (H5N1). As presented in Figure 11, the hemagglutinin gene coding sequence (Acc. #EF541394), with its native signal peptide and transmembrane domain, was first assembled in the plastocyanin expression cassette – promoter, 5'UTR, 3'UTR and transcription termination sequences from the alfalfa plastocyanin gene – and the assembled cassette (660) was inserted into to a pCAMBIA binary

plasmid. This plasmid was then transfected into *Agrobacterium* (AGL1), creating the recombinant strain AGL1/660, which was used for transient expression.

[00285] *N. benthamiana* plants were infiltrated with AGL1/660, and the leaves were harvested after a six-day incubation period. To determine whether H5 accumulated in the agroinfiltrated leaves, protein were first extracted from infiltrated leaf tissue and analyzed by Western blotting using anti-H5 (Vietnam) polyclonal antibodies. A unique band of approximately 72 kDa was detected in extracts (Figure 12), corresponding in size to the uncleaved HA0 form of influenza hemagglutinin. The commercial H5 used as positive control (A/Vietnam/1203/2004; Protein Science Corp., Meriden, CT, USA) was detected as two bands of approximately 48 and 28 kDa, corresponding to the molecular weight of HA1 and HA2 fragments, respectively. This demonstrated that expression of H5 in infiltrated leaves results in the accumulation of the uncleaved translation product.

[00286] The formation of active HA trimers was demonstrated by the capacity of crude protein extracts from AGL1/660-transformed leaves to agglutinate turkey red blood cells (data not shown).

Example 2: Characterization of hemagglutinin-containing structures in plant extracts using size exclusion chromatography

[00287] The assembly of plant-produced influenza hemagglutinin into high molecular weight structures was assessed by gel filtration. Crude protein extracts from AGL1/660-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). Elution fractions were assayed for their total protein content and for HA abundance using immunodetection with anti-HA antibodies (Figure 13A). As shown in Figure 13A, Blue Dextran (2 MDa) elution peaked early in fraction 10 while the bulk of host proteins was retained in the column and eluted between fractions 14 and 22. When proteins from 200 µL of each SEC elution fraction were concentrated (5-fold) by acetone-precipitation and analyzed by Western blotting (Figure 15A, H5), hemagglutinin (H5) was primarily found in fractions 9 to 14 (Figure 13B). Without wishing to be bound by theory, this suggests

that the HA protein had either assembled into a large superstructure or that it has attached to a high molecular weight structure.

[00288] A second expression cassette was assembled with the H1 nucleic acid sequence from A/New Caledonia/20/99 (H1N1) (SEQ ID NO: 33; Figure 16; GenBank Accession No. AY289929) to produce construct 540 (Figure 11). A chimeric gene construct was designed so as to produce a soluble trimeric form of H1 in which the signal peptide originated from a plant protein disulfide isomerase gene, and the transmembrane domain of H1 was replaced by the pII variant of the GCN4 leucine zipper, a peptide shown to self-assemble into trimers (Harbury et al., 1993) (cassette 544, figure 11). Although lacking the transmembrane domain, this soluble trimeric form was capable of hemagglutination (data not shown).

[00289] Protein extracts from plants infiltrated with AGL1/540 or AGL1/544 were fractionated by SEC and the presence of H1 eluted fractions was examined by Western blotting with anti-influenza A antibodies (Fitzgerald, Concord, MA, USA). In AGL1/540-infiltrated leaves, H1 accumulated mainly as a very high molecular weight structure, with the peak was skewed toward smaller size structures (H1; Figure 13C). In AGL1/544-infiltrated leaves, the soluble form of H1 accumulated as isolated trimers as demonstrated by the elution pattern from gel filtration which parallels the host protein elution profile (soluble H1; Figure 13D). In comparison, H1 rosettes (Protein Science Corp., Meriden, CT, USA), consisting in micelles of 5-6 trimers of hemagglutinin eluted at fractions 12 to 16 (Figure 13E), earlier than the soluble form of H1 (Figure 13D) and later than the native H1 (Figure 13C).

[00290] To evaluate the impact of M1 co-expression on hemagglutinin assembly into structure, a M1 expression cassette was assembled using the nucleic acid corresponding to the coding sequence of the A/PR/8/34 (H1N1) M1 (SEQ ID NO: 35; Figure 18; GenBank Accession No. NC_002016). The construct was named 750 and is presented in Figure 11. For the co-expression of M1 and H1, suspensions of AGL1/540 and AGL1/750 were mixed in equal volume before infiltration. Co-infiltration of multiple *Agrobacterium* suspensions permits co-expression of multiple transgenes. The Western blot analysis of SEC elution fractions shows that the co-expression of M1 did not modify the elution profile of the H1 structures, but resulted in a decrease in H1 accumulation in the agroinfiltrated leaves (see Figure 13F).

Example 3: Isolation of H5 structures by centrifugation in sucrose gradient and observation under electron microscopy

[00291] The observation of hemagglutinin structure under electron microscopy (EM) required a higher concentration and purity level than that obtained from SEC on crude leaf protein extracts. To allow EM observation of H5 structures, a crude leaf protein extract was first concentrated by PEG precipitation (20% PEG) followed by resuspension in 1/10 volumes of extraction buffer. The concentrated protein extract was fractionated by S-500 HR gel filtration and elution fractions 9, 10, and 11 (corresponding to the void volume of the column) were pooled and further isolated from host proteins by ultracentrifugation on a 20-60% sucrose density gradient. The sucrose gradient was fractionated starting from the top and the fractions were dialysed and concentrated on a 100 NMWL centrifugal filter unit prior to analysis. As shown on the Western blots and hemagglutination results (Figure 14A), H5 accumulated mainly in fractions 16 to 19 which contained $\approx 60\%$ sucrose, whereas most of the host proteins peaked at fraction 13. Fractions 17, 18, and 19 were pooled, negatively stained, and observed under EM. Examination of the sample clearly demonstrated the presence of spiked spheric structures ranging in size from 80 to 300 nm which matched the morphological characteristics of influenza VLPs (Figure 14B).

Example 4: Purification of influenza H5 VLPs from plant biomass

[00292] In addition to an abundant content of soluble proteins, plant leaf extracts contain a complex mixture of soluble sugars, nucleic acids and lipids. The crude extract was clarified by a pH shift and heat treatment followed by filtration on diatomaceous earth (see *Material and method* section for a detailed description of the clarification method). Figure 15A (lanes 1-4) presents a Coomassie Blue stained gel comparing protein content at the various steps of clarification. A comparison of protein content in the crude extract (lane 1) and in the clarified extract (lane 4) reveals the capacity of the clarification steps to reduce the global protein content and remove most of the major contaminant visible at 50 kDa in crude leaf extracts. The 50 kDa band corresponds to the RuBisCO large subunit, representing up to 30% of total leaf proteins.

[00293] Influenza H5 VLPs were purified from these clarified extracts by affinity chromatography on a fetuin column. A comparison of the load fraction (Figure 15A, lane 5) with the flowthrough (Figure 15A, lane 6) and the eluted VLPs (Figure 15A, lane 7) demonstrates the specificity of the fetuin affinity column for influenza H5 VLPs in plant clarified extract.

[00294] The purification procedure resulted in over 75% purity in H5, as determined by densitometry on the Coomassie Blue stained SDS-PAGE gel (Figure 15A, lane 7). In order to assess the structural quality of the purified product, the purified H5 was concentrated on a 100 NMWL (nominal molecular weight limit) centrifugal filter unit and examined under EM after negative staining. Figure 15B shows a representative sector showing the presence of profuse VLPs. A closer examination confirmed the presence of spikes on the VLPs (figure 15C).

[00295] As shown in Figure 15D, H5 VLPs were purified to approx. 89% purity from clarified leaf extract by affinity chromatography on a fetuin column, based on the density of the Coomassie Blue stained H5 hemagglutinin and on total protein content determination by the BCA method.

[00296] The bioactivity of HA VLPs was confirmed by their capacity to agglutinate turkey red blood cells (data not shown).

[00297] Figure 20B also confirms the identity of the purified VLP visualized by Western blotting and immunodetection with an anti-H5 polyclonal serum (A/Vietnam/1203/2004). A unique band of approximately 72 kDa is detected and corresponds in size to the uncleaved HA0 form of influenza hemagglutinin. Figure 15c shows the VLP structure of the vaccine with the hemagglutinin spikes covering its structure.

[00298] VLPs were formulated for immunization of mice by filtering through a 0.22 μ m filter; endotoxin content was measured using the endotoxin LAL (Limulus Amebocyte Lysate) detection kit (Lonza, Walkersville, MS, USA). The filtered vaccine contained $105,8 \pm 11,6\%$ EU/ml (endotoxin units/ml).

Example 5: Localization of influenza VLPs in plants

[00299] To localize the VLPs and confirm their plasma membrane origin, thin leaf sections of H5-producing plants were fixed and examined under TEM after positive staining. Observation of leaf cells indicated the presence of VLPs in extracellular cavities formed by the invagination of the plasma membrane (Figure 19).
5 The shape and position of the VLPs observed demonstrated that despite the apposition of their plasma membranes on the cell wall, plant cells have the plasticity required to produce influenza VLPs derived from their plasma membrane and accumulate them in the apoplastic space.

Example 6: Plasma Membrane Lipid analysis

10 [00300] Further confirmation of the composition and origin of the plant influenza VLPs was obtained from analyses of the lipid content. Lipids were extracted from purified VLPs and their composition was compared to that of highly purified tobacco plasma membranes by high performance thin layer chromatography (HP-TLC). The migration patterns of polar and neutral lipids from VLPs and control
15 plasma membranes were similar. Purified VLPs contained the major phospholipids (phosphatidylcholine and phosphatidylethanolamine) and sphingolipids (glucosylceramide) found in the plasma membrane (Figure 27A), and both contained free sterols as the sole neutral lipids (Figure 27B). However, immunodetection of a plasma membrane protein marker (ATPase) in purified VLP extracts showed that the VLP
20 lipid bilayer does not contain one of the major proteins associated with plant plasma membranes, suggesting that host proteins may have been excluded from the membranes during the process of VLPs budding from the plant cells (Figure 27C).

Example 7: Immunogenicity of the H5 VLPs and effect of route of administration

25 [00301] Mice were administered plant-made H5 VLPs by intramuscular injection, or intranasal (inhalation). 0.1 to 12 ug of VLPs were injected intramuscularly into mice, with alum as an adjuvant, according to the described methods. Peak antibody titers were observed with the lowest antigen quantity, in a similar magnitude to that of 5 ug recombinant, soluble hemagglutinin (HA) (Figure
30 20A).

[00302] 0.1 to 1 ug plant-made H5 VLPs were administered intranasally with a chitosan adjuvant provided for an antibody response greater than that of the recombinant soluble HA with an alum adjuvant (Figure 20B).

[00303] For both administration routes, and over a range of antigen quantities, seroconversion was observed in all of the mice tested. Recombinant H5 soluble antigen conferred low ($<1/40$) or negligible ($1<1/10$ for the non-adjuvanted recombinant H5) HI titres.

Example 8:Hemagglutination-inhibition antibody titer (HAI) H5 VLP

[00304] Figure 21 A, B illustrates the hemagglutination inhibition (HAI) antibody response 14 days following a “boost” with plant-made H5 VLP, or recombinant soluble HA. The lowest dose of antigen (0.1 ug) when administered intramuscularly produced a superior HAI response to a 10-fold greater administration (5 ug) of recombinant soluble HA. Increasing doses of H5 VLP provided a modest increase in HAI over the lowest dose.

[00305] HAI response following intranasal administration was significantly increased in mice administered plant-made H5 VLPs (1.0 or 0.1 ug) compared to those administered 1 ug recombinant soluble HA, which was similar to the negative control. All mice immunized by intramuscular injection of H5 VLPs (from 0.1 to 12 μ g) had higher HAI titers than mice immunised with the control HA antigen (Figure 4a – now 21A). For the same dose of 5 μ g, VLPs induced HAI titers 20 times higher than the corresponding dose of the control HA antigen. VLPs also induced significantly higher HAI titers than the control HA antigen when delivered through the intranasal route (Figure 21b). For a given dose of H5 VLP the levels of HAI titers were lower in mice immunised intranasally than for mice immunised intramuscularly; 1 μ g VLP induced a mean HAI titer of 210 when administered i.m. while the same dose induced a mean HAI titer of 34 administered i.n..

[00306] When administered intramuscularly, all doses of VLPs induced high level of antibodies capable of binding homologous whole inactivated viruses (Figures 20b and 24). No significant difference was found between the plant-made VLP vaccine and the control HA antigen (except the 12 μ g VLP group 14 days after boost),

as both antigen preparations induce high binding antibody titers against the homologous strain. However, when administered intranasally, VLPs induced higher binding antibody titers in than did the control HA antigen (Figure 20b). When mixed with Chitosan, immunization with one microgram VLP induced a reciprocal mean Ab titer of 5 500, 8.6 times higher than the level found in mice immunized with 1 µg of the control HA antigen (reciprocal mean Ab titer of 920).

[00307] The immunogenicity of the plant-derived influenza VLPs was then investigated through a dose-ranging study in mice. Groups of five BALB/c mice were immunized intramuscularly twice at 3-week intervals with 0.1 µg to 12 µg of VLPs containing HA from influenza A/Indonesia/5/05 (H5N1) formulated in alum (1:1 ratio). Hemagglutination-inhibition titers (HI), using whole inactivated virus antigen (A/Indonesia/5/05 (H5N1)), were measured on sera collected 14 days after the second immunization. Immunization with doses of VLP as low as 0.1 µg induced the production of antibodies that inhibited viruses from agglutinating erythrocytes at high dilutions (Figure 21A). Parallel immunization of mice with 5µg of non-VLP alum-adjuvanted control H5 antigen (also from A/Indonesia/5/05) induce an HI response that was 2-3 logs lower than that achieved with the lowest VLP dose.

[00308] For both administration routes, and over a range of antigen quantities, the HAI response is superior in mice administered VLPs.

Example 9: Effect of adjuvant on immunogenicity of H5 VLPs

[00309] Plant-made H5 VLPs have a plasma membrane origin (Figure 19, Example 5). Without wishing to be bound by theory, enveloped viruses or VLPs of enveloped viruses generally acquire their envelope from the membrane they bud through. Plant plasma membranes have a phytosterol complement that is rarely, if ever found in animal cells, and several of these sterols have been demonstrated to exhibit immunostimulatory effects.

[00310] Plant-made H5 VLPs were administered intramuscularly (Figure 22A) or intranasally (Figure 22B) to mice in the presence or absence of an adjuvant, and the HAI (hemagglutination inhibition antibody response) determined. VLPs, in the presence or absence of an added adjuvant (alum or chitosan, as in these examples) in

either system of administration demonstrated a significantly greater HAI hemagglutinin inhibition than recombinant soluble HA. Even in the absence of an added adjuvant (i.e. alum or chitosan), plant-made H5 VLPs demonstrate a significant HAI, indicative of a systemic immune response to administration of the antigen.

5 [00311] Alum enhanced the mean level of HAI titers by a factor of 5 for intramuscular administration of VLP (Figure 22a) and by a factor of 3.7 for the control HA antigen. When administered i.m., 5 µg VLPs induced a mean HAI titer 12 times higher than the corresponding dose of control HA antigen. Chitosan did not
10 boost the mean HAI level of the control HA antigen (Figure 22b) while it increased the mean HAI level of mice immunised with 1 µg VLP administered i.n. by a factor of 5-fold.

Example 10: Antibody isotypes

[00312] Mice administered plant-made H5 VLPs or recombinant soluble HA in the presence or absence of alum as an added adjuvant demonstrate a variety of
15 immunoglobulin isotypes (Figure 23A).

[00313] In the presence of an added adjuvant, the antibody isotype profiles of VLPs and the HA are similar, with IgG1 being the dominant isotype. When VLPs or HA are administered without an added adjuvant, IgG1 response is reduced, but remains the dominant isotype response to VLPs, with IgM, IgG2a, IgG2B and IgG3
20 maintaining similar titers as in the presence of an added adjuvant. IgG1, IgG2a, and IgG2b titers are markedly reduced when HA is administered without an added adjuvant.

[00314] These data, therefore, demonstrate that plant-made VLPs do not require an added adjuvant to elicit a antibody response in a host.

25 [00315] Antibody titers against whole inactivated influenza virus strains (A/Indonesia/5/05; A/Vietnam/1203/04)I in mice administered plant-made VLPs or soluble recombinant HA intramuscularly in the presence of an added antigen are illustrated in Figure 23B. No significant difference is observed in the antibody titers for these influenza strains in mice administered 1 ug or 5 ug of VLPs or 5 ug of
30 soluble HA.

Example 11: Cross-reactivity of serum antibodies induced by the H5 VLP vaccine

[00316] Cross-reactivity of serum antibodies induced by H5 VLP was assessed against whole inactivated influenza viruses of different strains. All VLP doses (from 0.1 to 12 µg) as well as 5 µg of control HA antigen induced high binding antibody
5 titers against a clade 1 strain (A/Vietnam/1194/04), the homologous strain A/Indonesia/5/05 of clade 2.1, and a clade 2.2 strain A/turkey/Turkey/1/05 (Figure 25A).

[00317] However, only the plant-made VLP induced HAI titer against the A/turkey/Turkey/1/05 strain (Figure 25b). HAI titers for the A/Indonesia/5/05 were
10 high for VLPs.

Example 12: Cross-protection conferred by immunization with plant-made H5 VLP

[00318] Mice that previously had been administered a two-dose regimen of A/Indonesia/5/05 H5 VLPs as described, were subsequently challenged intranasally
15 with influenza A/Turkey/582/06 (H5N1) ("Turkey H5N1") infectious virus, and observed. The dose administered, per animal, was 10 LD₅₀ (4.09 X 10⁵ CCID₅₀).

[00319] By 7 days post-challenge, only 37.5% of the mice administered the PBS vaccine control had survived exposure to Turkey H5N1 (Figure 26A). 100% of
20 animals administered the control antigen (HA) or 1, 5 or 15 µg of Indonesia H5 VLPs survived up to 17 days post-challenge, when the experiment was terminated.

[00320] Body mass of the mice was also monitored during the experiment, and the average mass of the surviving mice plotted (Figure 26B). Mice administered 1, 5 or 15 µg of the Indonesia H5 VLPs before challenge did not lose any appreciable
25 mass during the course of the experiment, and in particular mice administered 5 µg of the VLPs appear to have gained significant mass. Negative control mice (no Turkey H5N1 challenge) did not appreciably gain or lose body mass. Positive control mice (not administered VLPs, but challenged with Turkey H5N1) exhibited significant loss of body mass during the course of the experiment, and three of these mice died. As
30 body mass is an average of all mice in the cohort, removal of the 'sickest' mice (the 3 that died) may lead to an apparent overall increase in mass, however note that the

average body mass of the positive control cohort is still significantly below that of the negative or the VLP-treated cohorts.

[00321] These data, therefore, demonstrate that plant-made influenza VLPs comprising the H5 hemagglutinin viral protein induce an immune response specific for pathogenic influenza strains, and that virus-like particles may bud from a plant plasma membrane. .

[00322] These data, therefore, demonstrate that plants are capable of producing influenza virus-like particles, and also for the first time, that virus-like particles can bud from a plant plasma membrane.

[00323] Further, using the current transient expression technology, a first antigen lot was produced only 16 days after the sequence of the target HA was obtained. Under the current yields for H5 VLPs, and at an exemplary dose of 5 µg per subject, each kg of infiltrated leaf may produce ~20,000 vaccine doses. This unique combination of platform simplicity, surge capacity and powerful immunogenicity provides for, among other embodiments, a new method response in the context of a pandemic.

Example 13: Characterization of hemagglutinin-containing structures in plant extracts using size exclusion chromatography

[00324] The assembly of plant-produced influenza hemagglutinin of different subtypes into high molecular weight structures was assessed by gel filtration. Crude or concentrated protein extracts from AGL1/660-, AGL1/540-, AGL1/783-, AGL1/780- and AGL1/785-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 46, Blue Dextran (2 MDa) elution peaked early in fraction 10. When proteins from 200 µL of each SEC elution fraction were concentrated (5-fold) by acetone-precipitation and analyzed by Western blotting (Figure 46), hemagglutinins were primarily found in fractions 7 to 14, and are indicative of the incorporation of HA into VLPs. Without wishing to be bound by theory, this suggests that the HA protein had either assembled into a large

superstructure or that it has attached to a high molecular weight structure, irrespectively of the subtype produced.

Example 14: Transient expression of seasonal influenza virus hemagglutinin by agroinfiltration in *N. benthamiana* plants

5 [00325] The ability of the transient expression system to produce seasonal influenza hemagglutinins was determined through the expression of the H1 subtype from strains A/Brisbane/59/2007 (H1N1) (plasmid #774), A/New Caledonia/20/1999 (H1N1) (plasmid #540) and A/Solomon Islands/3/2006 (H1N1) (plasmid #775). The hemagglutinin gene coding sequences were first assembled in the plastocyanin
10 expression cassette – promoter, 5'UTR, 3'UTR and transcription termination sequences from the alfalfa plastocyanin gene – and the assembled cassettes were inserted into to a pCAMBIA binary plasmid. The plasmids were then transfected into *Agrobacterium* (AGL1), producing *Agrobacterium* strains AGL1/774, AGL1/540 and AGL1/775, respectively.

15 [00326] *N. benthamiana* plants were infiltrated with AGL1/774, AGL1/540 and AGL1/775, and the leaves were harvested after a six-day incubation period. To determine whether H1 accumulated in the agroinfiltrated leaves, protein were first extracted from infiltrated leaf tissue and analyzed by Western blotting using anti-H1 antibodies. A unique band of approximately 72 kDa was detected in extracts (Figure
20 47), corresponding in size to the uncleaved HA0 form of influenza hemagglutinin. This demonstrated that expression of different annual epidemic strains of hemagglutinin in infiltrated leaves results in the accumulation of the uncleaved translation product.

25 **Example 15: Transient expression of potential pandemic influenza virus hemagglutinin by agroinfiltration in *N. benthamiana* plants**

[00327] The ability of the transient expression system to produce potential influenza hemagglutinins was determined through the expression of the H5 subtype from strains A/Anhui/1/2005 (H5N1) (plasmid #781), A/Indonesia/5/2005 (H5N1)
30 (plasmid #660) and A/Vietnam/1194/2004 (H5N1) (plasmid #782). The

hemagglutinin gene coding sequences were first assembled in the plastocyanin expression cassette – promoter, 5'UTR, 3'UTR and transcription termination sequences from the alfalfa plastocyanin gene – and the assembled cassettes were inserted into to a pCAMBIA binary plasmid. The plasmids were then transfected into *Agrobacterium* (AGL1).

[00328] *N. benthamiana* plants were infiltrated with AGL1/781, AGL1/660 and AGL1/782, and the leaves were harvested after a six-day incubation period. To determine whether H5 accumulated in the agroinfiltrated leaves, protein were first extracted from infiltrated leaf tissue and analyzed by Western blotting using anti-H5 antibodies. A unique band of approximately 72 kDa was detected in extracts (Figure 48), corresponding in size to the uncleaved HA0 form of influenza hemagglutinin. This demonstrated that expression of different potential pandemic strains of hemagglutinin in infiltrated leaves results in the accumulation of the uncleaved translation product.

Example 16: Transient expression of H5 by agroinfiltration in *N. tabacum* plants

[00329] The ability of the transient expression system to produce influenza hemagglutinin in leaves of *Nicotiana tabacum* was analysed through the expression of the H5 subtype from strain A/Indonesia/5/2005 (H5N1) (plasmid #660). The hemagglutinin gene coding sequences were first assembled in the plastocyanin expression cassette – promoter, 5'UTR, 3'UTR and transcription termination sequences from the alfalfa plastocyanin gene – and the assembled cassettes were inserted into to a pCAMBIA binary plasmid. The plasmids were then transfected into *Agrobacterium* (AGL1).

[00330] *N. tabacum* plants were infiltrated with AGL1/660 and the leaves were harvested after a six-day incubation period. To determine whether H5 accumulated in the agroinfiltrated leaves, protein were first extracted from infiltrated leaf tissue and analyzed by Western blotting using anti-H5 antibodies. A unique band of approximately 72 kDa was detected in extracts (Figure 49), corresponding in size to the uncleaved HA0 form of influenza hemagglutinin. This demonstrated that

expression of hemagglutinin in infiltrated *N. tabacum* leaves results in the accumulation of the uncleaved translation product.

Example 17: Immunogenicity of plant-made H5N1 VLP vaccine from A/Indonesia/5/05 (H5N1) in ferrets

5 [00331] A dose escalation study in ferrets was performed to evaluate the immunogenicity of plant derived VLPs. In vitro cross-reactivity of serum antibody induced by the H5 VLP vaccine at 3 doses (1, 5 and 15 ug) was assessed by hemagglutination inhibition of three other H5N1 strains – A/turkey/Turkey/1/05 (clade 2.2), A/Vietnam/1194/04 (clade 1) and A/Anhui/5/05 (all whole, inactivated virus), using serum taken 14 days after the first dose of vaccine (Figure 50A), and 14 days after the 2nd dose (Figure 50 B). For all 3 dose concentrations, cross-reactivity is observed

Example 17: Analysis of the immunogenicity results according to CHMP criteria.

15 [00332] The EMEA's Committee for Medicinal Products for Human Use (CHMP) (<http://www.emea.europa.eu/htms/general/contacts/CHMP/CHMP.html>) sets out three criteria (applied following the second dose) for vaccine efficacy: 1 - Number of seroconversion or significant increase in HI titers (4-fold) >40%; 2 - Mean geometric increase of at least 2.5; 3 - proportion of subjects achieving an HI titer of 1/40 should be at least 70%. Analysis of these criteria in the ferret model is shown in Tables 8-11. (*) is indicative of meeting or exceeding the CHMP criteria. A summary of cross-immunogenicity analysis in relation to CHMP criteria for licensure is shown in Table 12.

25 [00333] Animals were assessed daily for body weight, temperature and overall condition. No sign of sickness or discomfort was recorded during the study. Body weight and temperature was within normal ranges during the study. The vaccine was safe and tolerated by the study animals.

V81270WO

Table 8: Data for homologous strain (A/Indonesia/5/05)

Day	Criteria	Study group								
		1 µg	1 µg adjuvanted	5 µg	5 µg adjuvanted	7.5 µg	15 µg	15 µg adjuvanted	30 µg	5 µg ITC
14 (post 1st inj.)	% 4-fold increase in HI titer	0%	100%	0%	100% *	20%	20%	80% *	0%	0%
	Mean geometric increase	0%	7.6	0%	15.6 *	1.3	1.2	11.2 *	0%	0%
	% of HI titer of 1/40	0%	60%	0%	100% *	20%	0%	80% *	0%	0%
	Mean HI titer		38		78			56		
35 (14 days post boost)	% 4-fold increase in HI titer	0%	100% *	0%	60% *	0%	0%	40% *	0%	0%
	Mean geometric increase	0%	10.8 *	0%	5.9 *	0.7	0%	4 *	0%	0%
	% of HI titer of 1/40	0%	100% *	0%	100% *	0%	0%	100% *	0%	0%
	Mean HI titer		411		465			217		

Table 9: Data for heterologous strain (A/Vietnam/1194/04)

Day	Criteria	Study group								
		1 µg	1 µg adjuvanted	5 µg	5 µg adjuvanted	7.5 µg	15 µg	15 µg adjuvanted	30 µg	5 µg ITC
14 (post 1st inj.)	% 4-fold increase in HI titer		0%		0%			0%		
	Mean geometric increase		1.2		1.2			1.3		
	% of HI titer of 1/40		0%		0%			0%		
35 (post boost)	% 4-fold increase in HI titer		60%		80% *			60%		
	Mean geometric increase		2.3		5.1 *			1.78		
	% of HI titer of 1/40		0%		80% *			20%		

Table 10: Data for heterologous strain (A/turkey/Turkey/1/05)

V81270WO

Day	Criteria	Study group						
		1 µg	1 µg adjuvanted	5 µg	5 µg adjuvanted	7.5 µg	15 µg	15 µg adjuvanted
14 (post 1st inj.)	% 4-fold increase in HI titer Mean geometric increase % of HI titer of 1/40		40% 1.9 40%		20% 1.7 20%			60% 2.8 40%
35 (post boost)	% 4-fold increase in HI titer Mean geometric increase % of HI titer of 1/40		80% * 10.6 * 100% *		100% * 20.8 * 100% *			80% * 7.7 * 100% *
							30 µg	5 µg ITC

Table 11: Data for heterologous strain (A/Anhui/5/05)

Day	Criteria	Study group						
		1 µg	1 µg adjuvanted	5 µg	5 µg adjuvanted	7.5 µg	15 µg	15 µg adjuvanted
14 (post 1st inj.)	% 4-fold increase in HI titer Mean geometric increase % of HI titer of 1/40		40% 1.8 20%		20% 1.3 20%			80% * 6.4 * 80% *
35 (post boost)	% 4-fold increase in HI titer Mean geometric increase % of HI titer of 1/40		100% * 11.8 * 100% *		100% * 14.4 * 80% *			60% * 3 * 80% *
							30 µg	5 µg ITC

Table 12: Summary of cross-immunogenicity analysis in relation to CHMP criteria for licensure.

Strain	Criteria	Study group		
		1 µg adjuvanted	5 µg adjuvanted	15 µg adjuvanted
A/turkey/Turkey/1/05 (clade 2.2)	% 4-fold increase in HI titer	80% *	100% *	80% *
	Mean geometric increase	10.6 *	20.8 *	7.7 *
	% of HI titer of 1/40	100% *	100% *	100% *
A/Anhui/1/05 (clade 2.3)	% 4-fold increase in HI titer	100% *	100% *	60% *
	Mean geometric increase	11.8 *	14.4 *	3 *
	% of HI titer of 1/40	100% *	80% *	80% *
A/Vietnam/1194/04 (clade 1)	% 4-fold increase in HI titer	60%	80% *	60%
	Mean geometric increase	2.3	7.1 *	1.78
	% of HI titer of 1/40	0%	80% *	20%

5 Example 18: Selection of heagglutinin nucleotide sequences

[00334] The nucleotide sequences of the HA were retrieved from an influenza sequence database (see URL: flu.lanl.gov), or the NCBI influenza virus resource (see URL: ncbi.nlm.nih.gov/genomes/FLU/FLU.html). For several of the HA nucleic acid sequences, multiple entries are listed in the databases (Table 13). Some variation is associated primarily with the culture system (Origin – MDCK, egg, unknown, viral RNA/clinical isolate); for example, the glycosylation site at position 194 (mature protein numbering) of the HA is absent when type B influenza virus is expressed in allantoic fluid of eggs (see also Chen et al., 2008). For some sequences, domains may be lacking (e.g. incomplete clones, sequencing artifacts, etc.). The hemagglutinin sequence may divided into 5 domains: signal peptide (SP), HA1, HA2, transmembrane (DTm) and cytoplasmic tail. Domains of a first sequence may be combined with a domain from a second existing sequence e.g. the signal peptide of a first strain sequence may be combined with the balance of the hemagglutinin coding sequence from a second strain to provide a complete coding sequence.

Table 13: Variation in Influenza subtypes for selected HA coding sequences

	Strain	Sequence database reference No.	Origin	SP	HA 1	HA2	DTm	Divergence
H1	A/Solomon Islands/3/2006	ISDN231558 (Vaccine rec.)	MDCK	Y	Y	Y	Y	189: R ou G, 220: K (MDCK) T(Egg), 249: Q (MDCK) R(Egg), 550: L (MDCK) R (Egg)
	A/Solomon Islands/3/2006	ISDN238190	Egg	Y	Y	Y	Y	189: R ou G, 220: K (MDCK) T(Egg), 249: Q (MDCK) R(Egg), 550: L (MDCK) R (Egg)
	A/Solomon Islands/3/2006	EU100724	?	Y	Y	Y	Y	189: R ou G, 220: K (MDCK) T(Egg), 249: Q (MDCK) R(Egg), 550: L (MDCK) R (Egg)
	A/Solomon Islands/3/2006	ISDN220951	MDCK	Y	Y	N	N	189: R ou G, 220: K (MDCK) T(Egg), 249: Q (MDCK) R(Egg), 550: L (MDCK) R (Egg)
	A/Solomon Islands/3/2006	ISDN220953	Egg	Y	Y	N	N	189: R ou G, 220: K (MDCK) T(Egg), 249: Q (MDCK) R(Egg), 550: L (MDCK) R (Egg)
	A/Solomon Islands/3/2006	EU124137	Egg	Y	Y	N	N	189: R ou G, 220: K (MDCK) T(Egg), 249: Q (MDCK) R(Egg), 550: L (MDCK) R (Egg)
	A/Solomon Islands/3/2006	EU124135	MDCK	Y	Y	N	N	189: R ou G, 220: K (MDCK) T(Egg), 249: Q (MDCK) R(Egg), 550: L (MDCK) R (Egg)
	A/Solomon Islands/3/2006	EU124177	MDCK	Y	Y	Y	Y	189: R ou G, 220: K (MDCK) T(Egg), 249: Q (MDCK) R(Egg), 550: L (MDCK) R (Egg)
H1	A/Brisbane/59/2007	ISDN282676	MDCK	Y	Y	Y		203: D/I/N D est le plus abondant chez les H1
	A/Brisbane/59/2007	ISDN285101	Egg	Y	Y	N	N	203: D/I/N D est le plus abondant chez les H1
	A/Brisbane/59/2007	ISDN285777	Egg	Y	Y	Y	Y	203: D/I/N D est le plus abondant chez les H1
	A/Brisbane/59/2007	ISDN282677	Egg	Y	Y	Y	Y	203: D/I/N D est le plus abondant chez les H1
H3	A/Brisbane/10/2007	ISDN274893	Egg	Y	Y	Y	Y	202: V/G, 210:L/P, 215: del Ala, 242: S/I

	Strain	Sequence database reference No.	Origin	SP	HA 1	HA2	DTm	Divergence
	A/Brisbane/10/2007	ISDN257648	MDCK	N	Y	Y	Y	202: V/G, 210:L/P, 215: del Ala, 242: S/I
	A/Brisbane/10/2007	ISDN256751	Egg	Y	Y	Y	Y	202: V/G, 210:L/P, 215: del Ala, 242: S/I
	A/Brisbane/10/2007	ISDN273757	Egg	Y	Y	Y	Y	202: V/G, 210:L/P, 215: del Ala, 242: S/I
	A/Brisbane/10/2007	ISDN273759	Egg	Y	Y	Y	Y	202: V/G, 210:L/P, 215: del Ala, 242: S/I
	A/Brisbane/10/2007	EU199248	Egg	N	Y	Y	Y	202: V/G, 210:L/P, 215: del Ala, 242: S/I
	A/Brisbane/10/2007	EU199366	Egg	Y	Y	Y	Y	202: V/G, 210:L/P, 215: del Ala, 242: S/I
	A/Brisbane/10/2007	ISDN257043	Egg	N	Y	Y	Y	202: V/G, 210:L/P, 215: del Ala, 242: S/I
	A/Brisbane/10/2007	EU199250	MDCK	N	Y	Y	Y	202: V/G, 210:L/P, 215: del Ala, 242: S/I
	A/Brisbane/10/2007	ISDN275357	Egg	N	Y	N	N	202: V/G, 210:L/P, 215: del Ala, 242: S/I
	A/Brisbane/10/2007	ISDN260430	Egg	N	Y	Y	Y	202: V/G, 210:L/P, 215: del Ala, 242: S/I

H3	A/Wisconsin/67/2005	ISDN131464 (vaccine rec.)	?	N	Y	Y	N	138: A/S 156: H/Q 186: G/V 196: H/Y
	A/Wisconsin/67/2005	DQ865947	?	N	Y	partial	N	138: A/S 156: H/Q 186: G/V 196: H/Y
	A/Wisconsin/67/2005	EF473424	?	N	Y	Y	N	138: A/S 156: H/Q 186: G/V 196: H/Y
	A/Wisconsin/67/2005	ISDN138723	Egg	N	Y	Y	Y	138: A/S 156: H/Q 186: G/V 196: H/Y
	A/Wisconsin/67/2005	EF473455	Egg	N	Y	Y	Y	138: A/S 156: H/Q 186: G/V 196: H/Y
	A/Wisconsin/67/2005	ISDN138724	?	N	Y	Y	Y	138: A/S 156: H/Q 186: G/V 196: H/Y

	Strain	Sequence database reference No.	Origin	SP	HA 1	HA2	DTm	Divergence
B	B/Malaysia /2506/2004	ISDN126 672 (vaccine rec.)	Egg	Y	Y	N	N	120 K/N 210 T/A
	B/Malaysia/2506/2004	EF566433	Egg	Y	Y	N	N	120 K/N 210 T/A
	B/Malaysia/2506/2004	ISDN231 265	Egg	Y	Y	Y	Y	120 K/N 210 T/A
	B/Malaysia/2506/2004	ISDN231 557	MDCK	Y	Y	Y	Y	120 K/N 210 T/A
	B/Malaysia/2506/2004	EF566394	MDCK	Y	Y	N	N	120 K/N 210 T/A
	B/Malaysia/2506/2004	EU12427 4	Egg	Y	Y	Y	Y	120 K/N 210 T/A
	B/Malaysia/2506/2004	EU12427 5	MDCK	Y	Y	Y	Y	120 K/N 210 T/A
	B/Malaysia/2506/2004	ISDN124 776	MDCK	Y	Y	N	N	120 K/N 210 T/A

B	B/Florida/4 /2006	ISDN261 649	Egg	Y	Y	Y	N	lacking glycosylation site at position 211; 10 amino acids of DTm/cytoplasmic tail
	B/Florida/4/2006	EU10060 4	MDCK	N	Y	N	N	
	B/Florida/4/2006	ISDN218 061	MDCK	N	Y	N	N	
	B/Florida/4/2006	ISDN285 778	Egg	Y	Y	Y	Y	Includes cytoplasmic tail

B	B/Brisbane /3/2007	ISDN256 628	Egg	N	Y	N	N	lacking glycosylation site at position 211
	B/Brisbane/3/2007	ISDN263 782	Egg	Y	Y	Y	Y	lacking glycosylation site at position 211
	B/Brisbane/3/2007	ISDN263 783	MDCK	Y	Y	Y	Y	

H5	A/Viet Nam/1194/2004	ISDN386 86 (Vaccine rec.)	?	Y	Y	Y	Y	
----	----------------------	---------------------------	---	---	---	---	---	--

	Strain	Sequence database reference No.	Origin	SP	HA 1	HA2	DTm	Divergence
	A/Viet Nam/1194/2004	AY651333	?	Y	Y	Y	Y	
	A/Viet Nam/1194/2004	EF541402	?	Y	Y	Y	Y	

H5	A/Anhui/1/2005	DQ37928 (vaccine rec.)	?	Y	Y	Y	Y	
	A/Anhui/1/2005	ISDN131465	Egg	Y	Y	Y	Y	

H7	A/Chicken/Italy/13474/1999	AJ91720	ARN gen	Y	Y	Y	Y	
----	----------------------------	---------	---------	---	---	---	---	--

H7	A/Equine/Prague/56	AB298277 (Lab reassortant)	?	Y	Y	Y	Y	152 (R/G) 169 (T/I) 208 (N/D) (glycosylation site abolished)
	A/Equine/Prague/56	X62552	?	Y	Y	Y	Y	

H9	A/Hong Kong/1073/1999	AJ404626	?	Y	Y	Y	Y	
	A/Hong Kong/1073/1999	AB080226	?	N	Y	N	N	

H2	A/Singapore/1/1957	AB296074	?	Y	Y	Y	Y	
	A/Singapore/1/1957	L20410	RNA	Y	Y	Y	Y	
	A/Singapore/1/1957	L11142	?	Y	Y	Y	Y	

H2	A/Japan/305/1957	L20406	?	Y	Y	Y	Y	
	A/Japan/305/1957	L20407	?	Y	Y	Y	Y	
	A/Japan/305/1957	CY014976	?	Y	Y	Y	Y	
	A/Japan/305/1957	AY209953	?	Y	Y	N	N	
	A/Japan/305/1957	J02127	?	Y	Y	Y	Y	

	Strain	Sequence database reference No.	Origin	SP	HA 1	HA2	DTm	Divergence
	A/Japan/305/1957	DQ508841	?	Y	Y	Y	Y	
	A/Japan/305/1957	AY643086	?	Y	Y	Y	N	
	A/Japan/305/1957	AB289337	?	Y	Y	Y	Y	
	A/Japan/305/1957	AY643085	?	Y	Y	Y	Y	
	A/Japan/305/1957	AY643087	Drug resistant	Y	Y	Y	N	

H6	A/Teal/Hong Kong/W312/1997 (H6N1)	AF250479	Egg	Y	Y	Y	Y	
----	-----------------------------------	----------	-----	---	---	---	---	--

Y, N – Yes, No, respectively

SP – presence of signal peptide sequence Y/N

HA1 – complete HA1 domain Y/N

HA2 – complete HA2 domain Y/N

5 DTm – complete transmembrane domain Y/N

Strain: H1 from A/Solomon Islands/3/2006

[00335] Eight amino acid sequences were compared, and variations identified. (Table 14). Position 171 exhibited a variation of glycine (G) or arginine (R) in some sequences.

10

Table 14: A/Solomon Islands/3/2006 amino acid variation

Amino acid #*	MDCK	Egg
212	K	T
241	Q	R
542	L	R

Numbering from the starting M

Strain: H1 from A/Brisbane/59/2007

[00336] Position 203 exhibited a variation of aspartic acid (D), isoleucine (I) or asparagine (N).

Strain: H3 from A/Brisbane/10/2007

- 5 [00337] Sequence variations were observed at 5 positions (Table 15). In position 215, a deletion is observed in two sampled sequences.

Table 15: H3 from A/Brisbane/10/2007 amino acid variation

	Origin	202, 210, 215, 235 242*				
ISDN274893	Egg	V	L	-	Y	I
ISDN273759	Egg	G	P	A	S	I
EU199248	Egg	G	P	A	S	I
EU199366	Egg	G	P	A	S	I
ISDN273757	Egg	V	L	-	S	S
ISDN257043	Egg	G	P	A	S	I
EU199250	MDCK	G	L	A	S	I
ISDN375357	Egg	G	P	A	S	I
ISDN260430	Egg	G	P	A	S	I
ISDN256751	Egg	G	P	A	S	I
ISDN257648	MDCK	G	L	A	S	I

*Numbering from the starting M

10

Strain: H3 from A/Wisconsin/67/2005

[00338] Sequence variations in this strain were observed at 4 positions (Table 16).

Table 16: H3 from A/Wisconsin/67/2005 amino acid variation

	Origin	138, 156, 186, 196
ISDN138724	Unknown	A H G H
DQ865947	Unknown	S H V Y
EF473424	Unknown	A H G H
ISDN138723	Egg	S Q V Y
ISDN131464	Unknown	A H G H
EF473455	Egg	A H G H

*Numbering from the mature protein

Strain: B from B/Malaysia/2506/2004

[00339] Variation at two positions is observed (Table 17). Position 120 is not a
 5 glycosylation site; position 210 is involved in glycosylation; this glycosylation is
 abolished following culture in eggs.

Table 17: Hemagglutinin from B/Malaysia/2506/2004 amino acid variation

Amino acid #*	MDCK	Egg
120	K	N
210	T	A

* Numbering from the middle of SP

10

Strain: hemagglutinin from B/Florida/4/2006; ISDN261649

[00340] Observed variations include amino acid sequence variation at position
 211, depending on the culture system. Asparagine (N) is found in sequences isolated
 from MDCK cells, while glutamic acid (D) is found in sequence isolated from eggs.
 15 Position 211 is a glycosylation site, and is abolished following culture in eggs.

Strain: H2 from A/Singapore/1/1957

[00341] Sequence variations were observed in 6 positions (Table 18).

Table 18: H2 from A/Singapore/1/1957 amino acid variation

	Origin	Amino acid No.					
		166	168	199\	236	238	358
L20410	Viral RNA	K	E	T	L	S	V
L11142	Unknown	E	G	K	L	S	I
AB296074	Unknown	K	G	T	Q	G	V
Consensus A/Japan/305/1957		K	G	T	Q/L	G	V

¹ Numbering from the mature protein

5 *Strains: H5 from A/Vietnam/1194/2004 and H5 from A/Anhui/1/2005*

[00342] There were no variations observed in the amino acid sequence upon aligning the primary sequences of either of these H5 strains.

Strain: H6 from A/Teal/Hong Kong/W312/1997

[00343] Only one entry was available for strain (AF250179).

10 *Strain: H7 from A/Equine/Prague/56*

[00344] A total of 2 sequence entries were found in the databases. The entry AB298877 was excluded as it is a laboratory reassortant.

Strain: H9 from A/Hong Kong/1073/1999; AJ404626

15 [00345] A total of 2 sequence entries were found in the databases. Only one was complete.

[00346] All citations are hereby incorporated by reference.

[00347] The present invention has been described with regard to one or more embodiments. However, it will be apparent to persons skilled in the art that a number

of variations and modifications can be made without departing from the scope of the invention as defined in the claims.

References:

- Aymard, H. M., M. T. Coleman, W. R. Dowdle, W. G. Laver, G. C. Schild, and R. G. Webster. 1973. Influenza virus neuraminidase-inhibition test procedures. Bull. W.H.O. 48: 199-202
- Bollag, D.M., Rozycki, M.D., and Edelstein, S.J. (1996) *Protein methods (2nd edition)*. Wiley-Liss, New York, USA.
- Bligh, E.G., & Dyer, W.J. *Can. J. Med. Sci.* **37**, 911-917 (1959).
- Chen, B.J., Leser, G.P., Morita, E., and Lamb R.A. (2007) Influenza virus hemagglutinin and neuraminidase, but not the matrix protein, are required for assembly and budding of plasmid-derived virus-like particles. *J. Virol.* **81**, 7111-7123.
- Chen Z, Aspelund A, Jin H. 2008 Stabilizing the glycosylation pattern of influenza B hemagglutinin following adaptation to growth in eggs. *Vaccine* vol 26 p 361-371
- Crawford, J. , Wilkinson, B. , Vosnesensky, A. , Smith, G. , Garcia, M. , Stone, H. , and Perdue, M. L. (1999). Baculovirus-derived hemagglutinin vaccines protect against lethal influenza infections by avian H5 and H7 subtypes. *Vaccine* **17**, 2265-2274.
- Darveau, A., Pelletier, A. & Perreault, J. PCR-mediated synthesis of chimeric molecules. *Methods Neurosc.* **26**, 77-85 (1995).
- Grgacic EVL, Anderson DA. Virus-like particles: passport to immune recognition. *Methods* **2006**; **40**: 60-65.
- Gillim-Ross, L., and Subbarao, K. (2006) Emerging respiratory viruses: challenges and vaccine strategies. *Clin. Microbiol. Rev.* **19**, 614-636.
- Gomez-Puertas, P., Mena, I., Castillo, M., Vivo, A., Perez-Pastrana, E. and Portela, A. (1999) Efficient formation of influenza virus-like particles: dependence on the expression level of viral proteins. *J. Gen. Virol.* **80**, 1635-1645.

- Gomez-Puertas, P., Albo, C., Perez-Pastrana, E., Vivo, A., and Portela, A. (2000) Influenza Virus protein is the major driving force in virus budding. *J Virol.* 74, 11538-11547.
- Hamilton, A., Voinnet, O., Chappell, L. & Baulcombe, D. Two classes of short
5 interfering RNA in RNA silencing. *EMBO J.* 21, 4671-4679 (2002).
- Höfgen, R. & Willmitzer, L. Storage of competent cells for *Agrobacterium* transformation. *Nucleic Acid Res.* 16, 9877 (1988).
- Harbury PB, Zhang T, Kim PS, Alber T. (1993) A switch between two-, three-, and four-stranded coiled coils in GCN4 leucine zipper mutants. *Science*; 262: 1401-1407)
- 10 Horimoto T., Kawaoka Y. Strategies for developing vaccines against h5N1 influenza a viruses. *Trends in Mol. Med.* 2006; 12(11):506-514.
- Huang Z, Elkin G, Maloney BJ, Beuhner N, Amtzen CJ, Thanavala Y, Mason HS. Virus-like particle expression and assembly in plants: hepatitis B and Norwalk viruses. *Vaccine.* 2005 Mar 7;23(15):1851-8.
- 15 Johansson, B. E. (1999). Immunization with influenza A virus hemagglutinin and neuraminidase produced in recombinant baculovirus results in a balanced and broadened immune response superior to conventional vaccine. *Vaccine* 17, 2073-2080.
- Latham, T. , and Galarza, J. M. (2001). Formation of wild-type and chimeric
20 influenza virus-like particles following simultaneous expression of only four structural proteins. *J. Virol.* 75,6154- 6165.
- Lefebvre, B. *et al. Plant Physiol.* **144**, 402-418 (2007).
- Leutwiler LS et al 1986. *Nucleic Acid Sresearch* 14910):4051-64
- 25 Liu, L & Lomonossoff, G.P. Agroinfection as a rapid method for propagating Cowpea mosaic virus-based constructs. *J. Virol. Methods* 105, 343-348 (2002).
- Macala, L.J., Yo, R.K. & Ando, S. *J Lipid Res.* **24**, 1243-1250 (1983)

- Mattanovich, D., Rüker, F., da Câmara Machado, A., Laimer, M., Regner, F., Steinkellner, H., Himmler, G., and Katinger, H. (1989) Efficient transformation of *Agrobacterium* spp. By electroporation. *Nucl. Ac. Res.* 17, 6747.
- 5 Mena, I., Vivo, A., Perez, E., and Portela, A. (1996) Rescue of synthetic chloramphenicol acetyltransferase RNA into influenza virus-like particles obtained from recombinant plasmids. *J. Virol.* 70, 5016-5024.
- Mongrand S, Morel J, Laroche J, Claverol S, Carde JP, Hartmann MA et al.. Lipid rafts in higher plant cells. *The Journal of Biological Chemistry* 2004; 279(35): 36277-36286.
- 10 Neumann, G., Watanabe, T., and Kawaoka, Y. (2000) Plasmid-driven formation of virus-like particles. *J. Virol.* 74, 547-551.
- Nayak DP, Reichl U. (2004) Neuraminidase activity assays for monitoring MDCK cell culture derived influenza virus. *J Virol Methods* 122(1):9-15.
- 15 Olsen, C. W. , McGregor, M. W. , Dybdahl-Sissoko, N. , Schram, B. R. , Nelson, K. M. , Lunn, D., Macklin, M. D. , and Swain, W. F. (1997). Immunogenicity and efficacy of baculovirus- expressed and DNA-based equine influenza virus hemagglutinin vaccines in mice. *Vaccine* 15, 1149-1156.
- Quan FS, Huang C, Compans RW, Kang SM. Virus-like particle vaccine induces protective immunity against homologous and heterologous strains of influenza virus. *Journal of Virology* 2007; 81(7): 3514-3524.
- 20 Rowe, T. et al. 1999. Detection of antibody to avian influenza a (h5N1) virus in human serum by using a cmbiation of serologic assays. *J. Clin Microbiol* 37(4):937-43
- Saint-Jore-Dupas C et al. 2007. From planta to pharma with glycosylation in the toolbox. *Trends in Biotechnology* 25(7) :317-23
- 25 Sambrook J, and Russell DW. *Molecular cloning: a laboratory manual*. Cold Spring Harbor, N.Y. Cold Spring Harbor Laboratory Press, 2001.

- Stockhaus J et al 1987. Analysis of cis-active sequences involved in the leaf-specific expression of a potato gene in transgenic plants. *Proceedings of the National Academy of Sciences U.S.S.* 84(22):7943-7947.
- 5 Stockhaus J et al 1989. Identification of enhancer elements in the upstream region of the nuclear photosynthetic gene ST-LS1. *Plant Cell.* 1(8):805-13.
- Suzuki, Y. (2005) Sialobiology of influenza. Molecular mechanism of host range variation of influenza viruses. *Biol. Pharm. Bull* 28, 399-408.
- Tsuji M., *Cell. Mol. Life Sci.*, 63 (2006); 1889-1898
- Wakefield L., G.G. Brownlee *Nuc Acid Res.* 17 (1989); 8569-8580.
- 10 Kendal, AP, Pereira MS, Skehel J. Concepts and procedures for laboratory-based influenza surveillance. Atlanta: CDC; 1982. p.B17-B35
- WHO. Manual on animal influenza diagnosis and surveillance. Department of communicable disease surveillance and response. World Health Organisation Global Influenza Program. 2002.
- 15 Skehel JJ and Wildy DC *Ann Rev Biochem* 2000 69:531-69
- Vaccaro L et al 2005. *Biophysical J.* 88:25-36.
- Gamblin, S.J., Haire, L.F., Russell, R.J., Stevens, D.J., Xiao, B., Ha, Y., Vasisht, N., Steinhauer, D.A., Daniels, R.S., Elliot, A., Wiley, D.C., Skehel, J.J. (2004) The structure and receptor binding properties of the 1918 influenza hemagglutinin.
- 20 *Science* 303: 1838-1842

WHAT IS CLAIMED IS:

1. A nucleic acid comprising a nucleotide sequence encoding an influenza hemagglutinin (HA) operatively linked to a regulatory region active in a plant.
2. The nucleic acid of claim 1, wherein the influenza hemagglutinin is selected from the group consisting of H1, H2, H3, H4, H5, H6, H7, H8, H9, H10, H11, H12, H13, H14, H15, and H16.
3. A method of producing influenza virus like particles (VLPs) in a plant comprising:
 - a) introducing the nucleic acid of claim 1 into the plant, or portion thereof, and
 - b) incubating the plant under conditions that permit the expression of the nucleic acid, thereby producing the VLPs.
4. The method of claim 3, wherein in the step of introducing (step a), the nucleic acid is transiently expressed in the plant.
5. The method of claim 3, wherein, in the step of introducing (step a), the nucleic acid is stably expressed in the plant.
6. The method of claim 3 further comprising a step of
 - c) harvesting the host and purifying the VLPs.
7. A virus like particle (VLP) comprising an influenza virus HA protein and one or more than one lipid derived from a plant.
8. The virus like particle (VLP) of claim 7, wherein the influenza HA protein is H5 Indonesia.
9. A composition comprising an effective dose of the VLP of claim 7 and a pharmaceutically acceptable carrier.
10. A method of inducing immunity to an influenza virus infection in a subject, comprising administering the virus like particle of claim 7.
11. The method of claim 10, wherein the virus like particle is administered to a subject orally, intradermally, intranasally, intramuscularly, intraperitoneally, intravenously, or subcutaneously.

12. A virus like particle (VLP) comprising an influenza virus HA bearing plant-specific N-glycans, or modified N-glycans.
13. A composition comprising an effective dose of the VLP of claim 12 and a pharmaceutically acceptable carrier.
- 5 14. A method of inducing immunity to an influenza virus infection in a subject, comprising administering the composition of claim 13.
15. The method of claim 11, wherein the composition is administered to a subject orally, intradermally, intranasally, intramuscularly, intraperitoneally, intravenously, or subcutaneously.