

Recombinant influenza virus-like particles (VLPs) produced in transgenic plants expressing hemagglutinin

[001] This application is a Continuation-in-Part of and claims priority from PCT Application No. PCT/CA2008/001281, filed July 11, 2008. This application also claims priority from Canadian Application No. 2,615,372 filed January 21, 2008; and United States Application No. 61/022,775 filed January 22, 2008.

FIELD OF INVENTION

[002] 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.

10 BACKGROUND OF THE INVENTION

[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 viruses that bud from the plasma membrane of infected mammalian and avian 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
20 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 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,
25 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 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 century. The Spanish flu, caused by an H1N1 virus, in 1918-1919 led to the deaths of over 50 million people worldwide between 5 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 10 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. 15 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 20 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 25 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 30 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.

[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 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 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 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.

[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 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 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 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; 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 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 M1 proteins from nucleocapsid-associated ribonucleoproteins (RNPs), which migrate into the cell nucleus for viral RNA 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 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 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 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 (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 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 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 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] PCT Publications WO 2004/098530 and WO 2004/098533 teach expression of

5 Newcastle Disease Virus HN or Avian Influenza A/turkey/Wisconsin/68 (H5N9) in transformed NT-1 (tobacco) cells in culture. Compositions comprising the plant cell culture-expressed polypeptides elicit varying immune responses in rabbits and chickens.

[0022] 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.

10 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
15 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.

[0023] 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
20 pandemic and of yearly epidemics, response time is crucial for vaccination. A simpler VLP production system, for example, one that relies on the expression of only one or a few viral proteins without requiring expression of non-structural viral proteins is desirable to accelerate the development of vaccines.

[0024] In order to protect the world population from influenza and to stave off future pandemics,
25 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.

SUMMARY OF THE INVENTION

[0025] It is an object of the invention to provide improved influenza virus like particles (VLPs).

[0026] According to the present invention there is provided a nucleic acid comprising a nucleotide sequence encoding an antigen from an enveloped virus operatively linked to a regulatory region active in a plant. The antigen may be an influenza hemagglutinin (HA).

5 [0027] The HA may comprise a native, or a non-native signal peptide; the non-native signal peptide may be a protein disulfide isomerase signal peptide.

[0028] The HA encoded by the nucleic acid may be a type A influenza, a type B influenza, or is a subtype of type A influenza, selected from the group comprising H1, H2, H3, H4, H5, H6, H7, H8, H9, H10, H11, H12, H13, H14, H15, and H16. In some aspects of the invention, the HA encoded by the nucleic acid may be from a type A influenza, and selected from the group
10 comprising H1, H2, H3, H5, H6, H7 and H9.

[0029] 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.

[0030] The method may further comprise the steps of harvesting the plant and purifying or separating the VLPs from the plant tissue.

20 [0031] The method may further comprise, in the step of introducing (step a), a nucleic acid comprising a nucleotide sequence encoding one or more than one chaperon protein.

[0032] The one or more than one chaperone proteins may be selected from the group comprising Hsp40 and Hsp70.

25 [0033] 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.

[0034] According to another aspect of the present invention, there is provided a method of producing influenza virus like particles (VLPs) in a plant comprising providing a plant, or a

portion of a plant, comprising a nucleic acid comprising a nucleotide sequence encoding an antigen from an enveloped virus operatively linked to a regulatory region active in a plant., and incubating the plant or portion of the plant under conditions that permit the expression of the nucleic acid, thereby producing the VLPs.

- 5 [0035] The method may further comprise the steps of harvesting the plant and purifying or separating the VLPs from the plant tissue.

[0036] The present invention includes the above method, wherein following the step of providing, a nucleic acid comprising a nucleotide sequence encoding one or more than one chaperone protein operatively linked to a regulatory region active in a plant is introduced, and
10 the plant or portion of the plant incubated under conditions that permit expression of the nucleic acid, thereby producing the VLPs.

[0037] The one or more than one chaperone proteins may be selected from the group comprising Hsp40 and Hsp70.

[0038] The present invention includes the above method wherein, in the step of introducing (step
15 a), the nucleic acid encoding the HA is stably expressed in the plant. Furthermore, the VLPs may be purified using size exclusion chromatography.

[0039] The present invention also provides a virus like particle (VLP) comprising an influenza virus HA protein and one or more than one lipid derived from a plant.

[0040] The HA protein of the VLP may be of a type A influenza, a type B influenza, or is a
20 subtype of type A influenza HA selected from the group consisting of H1, H2, H3, H4, H5, H6, H7, H8, H9, H10, H11, H12, H13, H14, H15, and H16. In some aspects of the invention, the HA is from a type A influenza, selected from the group comprising H1, H2, H3, H5, H6, H7 and H9.

[0041] Also included in the present invention is a composition comprising an effective dose of a
25 VLP, the VLP comprising an influenza virus HA protein, one or more than one plant lipid, and a pharmaceutically acceptable carrier.

[0042] The present invention also contemplates fragments or portions of HA proteins that form VLPs in a plant.

[0043] The present invention also pertains to a VLP comprising an influenza virus HA bearing plant-specific N-glycans, or modified N-glycans. The HA protein of the VLP may be of a type A influenza, a type B influenza, or is a subtype of type A influenza HA selected from the group consisting of H1, H2, H3, H4, H5, H6, H7, H8, H9, H10, H11, H12, H13, H14, H15, and H16.

5 In some aspects of the invention, the HA is from a type A influenza, selected from the group comprising H1, H2, H3, H5, H6, H7 and H9.

[0044] 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

10 (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,
15 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
20 (H7N7), A/HongKong/1073/99 (H9N2)).

[0045] In an aspect of the invention, the HA protein may be an H1, H2, H3, H5, H6, H7 or H9 subtype. In an 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

25 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
30 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, H9 or B subtypes include SEQ ID NOs: 48-59.

[0046] The influenza virus HA protein may be H5 Indonesia.

[0047] The present invention also provides nucleic acid molecules comprising sequences

5 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, H3, H4, H5, H6, H7, H8, H9, H10, H11, H12, H13, H14, H15, H16, B or C. In another aspect of the invention, the HA protein encoded by the nucleic acid molecule may be an H1, H2, H3, H5, H6, H7, H9, or B subtype.

10 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 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

15 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), 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.

20 Additionally, the H9 protein encoded by the nucleic acid molecule may be from the A/HongKong/1073/99 (H9N2) strain. The HA protein from B subtype encoded by the nucleic acid may be from the B/Florida/4/2006, or B/Malaysia/2506/2004 strain. Examples of sequences of nucleic acid molecules encoding such HA proteins from H1, H2, H3, H5, H6, H7, H9 or B subtypes include SEQ ID NOs: 36-47 and 60-73.

25 [0048] The nucleic acid sequence may encode the influenza virus HA protein H5 Indonesia.

[0049] 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-

30 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.

[0050] 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.

[0051] 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.

[0052] 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.

[0053] 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 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) 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.

[0054] The present invention pertains to 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 VLP produced using a non-sialyating 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 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.

[0055] The present invention provides a method for producing VLPs containing 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 cells (generally referred to as "yeast lipids").

[0056] The present invention also pertains to a plant, plant tissue or plant cell comprising a nucleic acid comprising a nucleotide sequence encoding an antigen from an enveloped virus operatively linked to a regulatory region active in a plant. The antigen may be an influenza hemagglutinin (HA).

[0057] The plant may further comprise a nucleic acid comprising a nucleotide sequence encoding one or more than one chaperone proteins operatively linked to a regulatory region active in a plant. The one or more than one chaperone proteins may be selected from the group comprising Hsp40 and Hsp70.

[0058] 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).

- 5 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.

[0059] 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
10 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.

[0060] 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
15 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.

[0061] 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
20 (neuraminidase), to ensure VLP production in plants.

[0062] 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.

[0063] This summary of the invention does not necessarily describe all features of the invention.

25 **BRIEF DESCRIPTION OF THE DRAWINGS**

[0064] 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:

[0065] **FIGURE 1A** shows a sequence of an alfalfa plastocyanin-based expression cassette used for the expression of H1 from strain A/New Caledonia/20/99 (H1N1) 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
5 disulfide bridge.

[0066] **FIGURE 2A** shows a representation of plasmid 540 assembled for the expression of HA subtype H1 from strain A/New Caledonia/20/99 (H1N1). **FIGURE 2B** shows a representation of plasmid 660 assembled for the expression of HA subtype H5 from strain A/Indonesia/5/2005 (H5N1).

10 [0067] **FIGURE 3** shows a size exclusion chromatography of protein extracts from leaves producing hemagglutinin H1 or H5. **FIGURE 3A** show the elution profile of Blue Dextran 2000 (triangles) and proteins (diamonds). **FIGURE 3B** shows immunodetection (western blot; anti H1) of H1 (A/New Caledonia/20/99 (H1N1)) elution fractions following size exclusion chromatography (S500HR beads). **FIGURE 3C** show the elution profile of H5; Blue Dextran
15 2000 (triangles) and proteins (diamonds). **FIGURE 3D** shows immunodetection (western blot; anti H5) of H5 (A/Indonesia/5/2005 (H5N1)) elution fractions following size exclusion chromatography (S500HR beads).

[0068] **FIGURE 4A** shows the sequence encoding the N terminal fragment of H1 (A/New Caledonia/20/99 (H1N1)) (SEQ ID NO:1). **FIGURE 4B** shows the sequence encoding the C
20 terminal fragment of H1 (A/New Caledonia/20/99 (H1N1)) (SEQ ID NO:2).

[0069] **FIGURE 5** shows the complete sequence encoding HA0 of H1 (A/New Caledonia/20/99 (H1N1)) (SEQ ID NO:28).

[0070] **FIGURE 6** shows the sequence encoding H5 (A/Indonesia/5/2005 (H5N1)) flanked by a HindIII site immediately upstream of the initial ATG, and a SacI site immediately downstream
25 of the stop (TAA) codon (SEQ ID NO:3)

[0071] **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).

[0072] **FIGURE 8A** shows the amino acid sequence of the H1 (A/New Caledonia/20/99 (H1N1)) peptide sequence (SEQ ID NO:9). **FIGURE 8B** shows the amino acid sequence of H5 (A/Indonesia/5/2005 (H5N1)) peptide sequence (SEQ ID NO:10). Native signal peptide is indicated in bold.

5 [0073] **FIGURE 9** shows the nucleotide sequence of HA of influenza A subtype H7 (SEQ ID No: 11).

[0074] **FIGURE 10A** shows the nucleotide sequence of Influenza A HA, subtype H2 (SEQ ID NO:12). **FIGURE 10B** shows the nucleotide sequence of Influenza A HA subtype H3 (SEQ ID NO:13). **FIGURE 10C** shows the nucleotide sequence of Influenza A HA subtype H4 (SEQ ID
10 NO:14). **FIGURE 10D** shows the nucleotide sequence of Influenza A HA subtype H5 (SEQ ID NO:15). **FIGURE 10E** shows the nucleotide sequence of Influenza A HA subtype H6 (SEQ ID NO:16). **FIGURE 10F** shows the nucleotide sequence of Influenza A HA subtype H8 (SEQ ID NO:17). **FIGURE 10G** shows the nucleotide sequence of Influenza A HA subtype H9 (SEQ ID NO:18). **FIGURE 10H** shows the nucleotide sequence of Influenza A HA subtype H10 (SEQ ID
15 NO:19). **FIGURE 10I** shows the nucleotide sequence of Influenza A HA subtype H11 (SEQ ID NO:20). **FIGURE 10J** shows the nucleotide sequence of Influenza A HA subtype H12 (SEQ ID NO:21). **FIGURE 10K** shows the nucleotide sequence of Influenza A HA subtype H13 (SEQ ID NO:22). **FIGURE 10L** shows the nucleotide sequence of Influenza A HA subtype H14 (SEQ ID NO:23). **FIGURE 10M** shows the nucleotide sequence of Influenza A HA subtype H15 (SEQ ID
20 NO:24). **FIGURE 10N** shows the nucleotide sequence of Influenza A HA subtype H16 (SEQ ID NO:25). **FIGURE 10O** shows the nucleotide sequence of Influenza B HA (SEQ ID NO:26). **FIGURE 10P** shows the nucleotide sequence of Influenza C HA (SEQ ID NO:27). **FIGURE 10Q** shows the nucleotide sequence of primer XmaI-pPlas.c (SEQ ID NO: 29). **FIGURE 10R** shows the nucleotide sequence of primer SacI-ATG-pPlas.r (SEQ ID NO: 30). **FIGURE 10S** shows the
25 nucleotide sequence of primer SacI-PlasTer.c (SEQ ID NO: 31). **FIGURE 10T** shows the nucleotide sequence of primer EcoRI-PlasTer.r (SEQ ID NO: 32).

[0075] **FIGURE 11** shows a schematic representation of several constructs as used herein. Construct 660 comprises the nucleotide sequence to encode the HA subtype H5 (A/Indonesia/5/2005 (H5N1)) under operatively linked to the plastocyanin promoter (plasto) and
30 terminator (Pter); construct 540 comprises the nucleotide sequence to encode the HA subtype H1 (A/New Caledonia/20/99 (H1N1)) 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 (A/New Caledonia/20/99 (H1N1)), 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.

[0076] **FIGURE 12** shows immunodetection of H5 (A/Indonesia/5/2005 (H5N1)), 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).

[0077] **FIGURE 13** shows characterization of hemagglutinin structures by size exclusion chromatography. Protein extract from separate biomasses producing H5 (A/Indonesia/5/2005 (H5N1)), H1 (A/New Caledonia/20/99 (H1N1)), soluble H1, or H1 and M1 were separated by gel filtration on S-500 HR. Commercial H1 (A/New Caledonia/20/99 (H1N1)) 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.

[0078] **FIGURE 14** shows concentration of influenza H5 (A/Indonesia/5/2005 (H5N1)) 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.

[0079] **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, flowthrough; 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).

[0080] **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)

[0081] **FIGURE 17** shows a nucleotide sequence for *Medicago sativa* mRNA for protein disulfide isomerase. GenBank Accession No. Z11499 (SEQ ID NO: 34).

[0082] **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).

[0083] **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.

[0084] **FIGURE 20** shows induction of serum antibody responses 14 days after boost in Balb/c mice vaccinated with plant-made influenza H5 VLP (A/Indonesia/5/2005 (H5N1)) or recombinant soluble H5 (A/Indonesia/5/2005 (H5N1)). 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 (ln) of reciprocal end-point titers of five mice per group. Bars represent *mean deviation*. * $p < 0.05$ compared to recombinant soluble H5.

[0085] **FIGURE 21** shows hemagglutination inhibition antibody response (HAI) 14 days after boost in Balb/c mice vaccinated with plant-made influenza H5 VLP (A/Indonesia/5/2005 (H5N1)) or recombinant soluble H5 (A/Indonesia/5/2005 (H5N1)). **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 (ln) 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 H5.

[0086] **FIGURE 22** shows the effect of adjuvant on immunogenicity of the VLPs in Balb/c 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 (ln) of reciprocal end-point titers of five mice per group. Bars represent *mean deviation*. * $p < 0.05$ compared to the corresponding recombinant soluble H5.

[0087] **FIGURE 23** shows antibody response to H5 VLP (A/Indonesia/5/2005 (H5N1)) administration. **FIGURE 23(A)** Anti-Indonesia/5/05 immunoglobulin isotype in mice immunized through intramuscular administration, 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 H5N1 (A/Indonesia/5/2005) viruses as the coating agent. Bars represent *mean deviation*. * $p < 0.05$, ** $p < 0.001$ compared to the corresponding recombinant soluble H5 (A/Indonesia/5/2005 (H5N1)). **FIGURE 23(B)** Antibody titers against whole inactivated viruses (A/Indonesia/5/2005 (H5N1) and (A/Vietnam/1194/04 (H5N1))). All groups are statistically different to negative control.

[0088] **FIGURE 24** shows antibody titer against homologous whole inactivated viruses (A/Indonesia/5/05), 14 days weeks after first dose (week 2), 14 days after boost (week 5) or 30 days after boost (week 7) from Balb/c mice immunized with H5 VLP (A/Indonesia/5/2005 (H5N1)). GMT: geometric mean titer. Values are the GMT (ln) of reciprocal end-point titers of five mice per group. * $p < 0.05$ compared to recombinant soluble H5.

[0089] **FIGURE 25** shows *in vitro* cross-reactivity of serum antibodies from Balb/c mice immunized with H5 VLP (A/Indonesia/5/2005 (H5N1)) 30 days after boost. (A) Antibody titers

whole inactivated viruses. (B) Hemagglutination-inhibition titers against various whole inactivated viruses. Values are the GMT (ln) of reciprocal end-point titers of five mice per group. Bars represent *mean deviation*. All groups are statistically different to negative control. * $p < 0.05$ compared to the corresponding recombinant soluble H5. All values less than 10 were given an arbitrary value of 5 (1.6 for ln) and are considered negative.

[0090] **FIGURE 26** shows efficacy of the plant made H5 VLP (A/Indonesia/5/2005 (H5N1)). (A) Survival rate of mice after challenge with 1000 LD₅₀ (4.09×10^6 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

[0091] **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.

[0092] **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.

[0093] **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.

[0094] **FIGURE 30** shows the sequence spanning from DraIII to SacI sites of clone 776 - nucleotide sequence of A/Brisbane 10/2007 (H3N2) (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.

[0095] **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.

[0096] **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.

[0097] **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. Restriction sites are underlined; ATG is in bold and underlined.

[0098] **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. Restriction sites are underlined; ATG is in bold and underlined.

[0099] **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. Restriction sites are underlined; ATG is in bold and underlined.

[00100] **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. Restriction sites are underlined; ATG is in bold and underlined.

[00101] **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. Restriction sites are underlined; ATG is in bold and underlined.

[00102] **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. Restriction sites are underlined; ATG is in bold and underlined.

[00103] **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. Restriction sites are underlined; ATG is in bold and underlined.

[00104] **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 clone 775 (A/Solomon Islands 3/2006 (H1N1)). The open reading frame of clone 775 starts with the ATG indicated in Figure 29.

[00105] **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 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.

[00106] **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 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
5 reading frame of clone 779 starts with the ATG indicated in Figure 33.

[00107] **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 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
10 open reading frame of clone 781 starts with the ATG indicated in Figure 35.

[00108] **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
15 (A/Teal/HongKong/W312/97 (H6N1)). The open reading frame of clone 783 starts with the ATG indicated in Figure 37.

[00109] **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
20 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.

[00110] **FIGURE 46** shows immunodetection (western blot) of elution fractions 7-17 of plant-produced VLPs, following size exclusion chromatography. The elution peak (fraction 10) of BlueDextran is indicated by the arrow. Hemagglutinin subtypes H1, H2, H3, H5, H6 and H9
25 are shown. Hemagglutinin is detected in fractions 7-14, corresponding to the elution of VLPs.

[00111] **FIGURE 47** shows an immunoblot analysis of expression of a series of hemagglutinin from annual epidemic strains. Ten and twenty micrograms of leaf protein extracts were loaded in lanes 1 and 2, respectively, for plants expressing HA from various influenza strains (indicated at the top of the immunoblots).

[00112] **FIGURE 48A** shows an immunoblot analysis of expression of a series of H5 hemagglutinins from potential pandemic strains. Ten and twenty micrograms of protein extracts were loaded in lanes 1 and 2, respectively. **FIGURE 48B** shows an immunoblot analysis of expression of H2, H7 and H9 hemagglutinin from selected influenza strains. Ten and twenty
5 micrograms of protein extracts were loaded in lanes 1 and 2, respectively.

[00113] **FIGURE 49** shows an immunoblot of H5 from strain A/Indonesia/5/2005 in protein extracts from *Nicotiana tabacum* leaves, agroinfiltrated with AGL1/660. Two plants (plant 1 and plant 2) were infiltrated and 10 and 20 µg of soluble protein extracted from each plant were loaded in lanes 1 and 2, respectively.

10 [00114] **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 (A/Indonesia/5/2005 (H5N1)). 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
15 A/Indonesia/5/05. Values are the GMT (log₂) 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*.

[00115] **FIGURE 51** shows the nucleic acid sequence (SEQ ID NO: 60) of an HA
20 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

[00116] **FIGURE 52** shows the nucleic acid sequence (SEQ ID NO: 61) of an HA
25 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

[00117] **FIGURE 53** shows the nucleic acid sequence (SEQ ID NO: 62) 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
30 terminator sequences.

[00118] **FIGURE 54** shows the nucleic acid sequence (SEQ ID NO: 63) 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.

5 [00119] **FIGURE 55** shows the nucleic acid sequence (SEQ ID NO: 64) 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.

10 [00120] **FIGURE 56** shows the nucleic acid sequence (SEQ ID NO: 65) 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

15 [00121] **FIGURE 57** shows the nucleic acid sequence (SEQ ID NO: 66) 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

20 [00122] **FIGURE 58** shows the nucleic acid sequence (SEQ ID NO: 67) 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.

[00123] **FIGURE 59** shows the nucleic acid sequence (SEQ ID NO: 68) 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.

25 [00124] **FIGURE 60** shows the nucleic acid sequence (SEQ ID NO: 69) 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.

[00125] **FIGURE 61** shows the nucleic acid sequence (SEQ ID NO: 70) 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.

5 [00126] **FIGURE 62** shows the nucleic acid sequence (SEQ ID NO: 71) 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.

[00127] **FIGURE 63** shows the nucleic acid sequence (SEQ ID NO: 72) 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.

[00128] **FIGURE 64** shows the nucleic acid sequence (SEQ ID NO: 73) 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.

[00129] **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 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.

25 [00130] **FIGURE 66** shows amino acid sequence (SEQ ID NO: 75) of H1 New Caledonia (AAP34324.1) encoded by SEQ ID NO: 33.

[00131] **FIGURE 67** shows the amino acid sequence (SEQ ID NO: 76) of H1 Puerto Rico (NC_0409878.1) encoded by SEQ ID NO: 35

[00132] **FIGURE 68** shows the nucleic acid sequence of a portion of expression cassette number 828, from PacI (upstream promoter) to AscI (immediately downstream NOS terminator). CPMV HT 5'UTR sequence underlined with mutated ATG. ApaI restriction site (immediately upstream of ATG of protein coding sequence to be express, in this case C5-1 kappa light chain.)

5 [00133] **FIGURE 69** shows the nucleic acid sequence of a portion of construct number 663, from HindIII (in the multiple cloning site, upstream of the plastocyanin promoter) to EcoRI (immediately downstream of the plastocyanin terminator). H5 (from A/Indonesia/5/2005) coding sequence in fusion with PDI SP is underlined.

[00134] **FIGURE 70** shows the nucleic acid sequence of a portion of construct number
10 787, from HindIII (in the multiple cloning site, upstream of the plastocyanin promoter) to EcoRI (immediately downstream of the plastocyanin terminator). H1 (from A/Brisbane/59/2007) coding sequence in fusion with PDI SP is underlined.

[00135] **FIGURE 71** shows the nucleic acid sequence of a portion of construct number
15 790, from HindIII (in the multiple cloning site, upstream of the plastocyanin promoter) to EcoRI (immediately downstream of the plastocyanin terminator). H3 (from A/Brisbane/10/2007) coding sequence in fusion with PDI SP is underlined.

[00136] **FIGURE 72** shows the nucleic acid sequence of a portion of construct number
20 798, from HindIII (in the multiple cloning site, upstream of the plastocyanin promoter) to EcoRI (immediately downstream of the plastocyanin terminator). HA from B/Florida/4/2006 coding sequence in fusion with PDI SP is underlined.

[00137] **FIGURE 73** shows the nucleic acid sequence of a portion of construct number 580, from PacI (upstream of the 35S promoter) to AscI (immediately downstream of the NOS terminator). Coding sequence of H1 (from A/New Caledonia/20/1999) in fusion with PDI SP is underlined.

25 [00138] **FIGURE 74** shows the nucleic acid sequence of a portion of construct number 685, from PacI (upstream of the 35S promoter) to AscI (immediately downstream of the NOS terminator). Coding sequence of H5 from A/Indonesia/5/2005 is underlined.

[00139] **FIGURE 75** shows the nucleic acid sequence of a portion of construct number 686, from PacI (upstream of the 35S promoter) to AscI (immediately downstream of the NOS

terminator). Coding sequence of H5 from A/Indonesia/5/2005 in fusion with PDI SP is underlined.

[00140] **FIGURE 76** shows the nucleic acid sequence of a portion of construct number 732, from PacI (upstream of the 35S promoter) to AscI (immediately downstream of the NOS terminator). Coding sequence of H1 from A/Brisbane/59/2007 is underlined.

[00141] **FIGURE 77** shows the nucleic acid sequence of a portion of construct number 733, from PacI (upstream of the 35S promoter) to AscI (immediately downstream of the NOS terminator). Coding sequence of H1 from A/Brisbane/59/2007 in fusion with PDI SP is underlined.

[00142] **FIGURE 78** shows the nucleic acid sequence of a portion of construct number 735, from PacI (upstream of the 35S promoter) to AscI (immediately downstream of the NOS terminator). Coding sequence of H3 from A/Brisbane/10/2007 is underlined.

[00143] **FIGURE 79** shows the nucleic acid sequence of a portion of construct number 736, from PacI (upstream of the 35S promoter) to AscI (immediately downstream of the NOS terminator). Coding sequence of H3 from A/Brisbane/10/2007 in fusion with PDI SP is underlined

[00144] **FIGURE 80** shows the nucleic acid sequence of a portion of construct number 738, from PacI (upstream of the 35S promoter) to AscI (immediately downstream of the NOS terminator). Coding sequence of HA from B/Florida/4/2006 is underlined.

[00145] **FIGURE 81** shows the nucleic acid sequence of a portion of construct number 739, from PacI (upstream of the 35S promoter) to AscI (immediately downstream of the NOS terminator). Coding sequence of HA from B/Florida/4/2006 in fusion with PDI SP is underlined.

[00146] **FIGURE 82** shows a nucleic acid sequence encoding Msj1 (SEQ ID NO: 114).

[00147] **FIGURE 83** shows the nucleic acid sequence of a portion of construct number R850, from HindIII (in the multiple cloning site, upstream of the promoter) to EcoRI (immediately downstream of the NOS terminator). HSP40 coding sequence is underlined.

[00148] **FIGURE 84** shows the nucleic acid sequence of a portion of construct number R860, from HindIII (in the multiple cloning site, upstream of the promoter) to EcoRI (immediately downstream of the NOS terminator). HSP70 coding sequence is underlined.

[00149] **FIGURE 85** shows the nucleic acid sequence of a portion of construct number R870, from HindIII (in the multiple cloning site, upstream of the promoter) to EcoRI (immediately downstream of the NOS terminator). HSP40 coding sequence is in underlined italic and HSP70 coding sequence is underlined. A) nucleotides 1-5003; B) nucleotides 5004-9493.

[00150] **FIGURE 86** shows a schematic representation of construct R472.

[00151] **FIGURE 87** shows an immunoblot analysis of expression of HA using a signal peptide from alfalfa protein disulfide isomerase. Twenty micrograms of leaf protein extract obtained from 3 separate plants were loaded on the SDS-PAGE except for the H1 (A/New Caledonia/20/99 (H1N1)) where five micrograms were used. The indicated controls (whole inactivated virus (WIV) of homologous strain) were spiked in five or twenty micrograms of mock-infiltrated plants. a) Expression of H1 from A/New Caledonia/20/99), b) expression of H1 from A/Brisbane/59/2007, c) expression of H3 from A/Brisbane/10/2007, d) expression of H5 from A/Indonesia/5/2005, e) expression of HA from B/Florida/4/2006. The arrows indicate the immunoband corresponding to HA0. SP WT: native signal peptide, PS PDI: alfalfa PDI signal peptide.

[00152] **FIGURE 88** shows a comparison of HA expression strategies by immunoblot analysis of leaf protein extracts. HA was produced using plastocyanin- or CPMV-HT-based cassettes. For CPMV-HT, the wild-type HA signal peptide and the signal peptide from alfalfa PDI were also compared. Twenty micrograms of protein extract were loaded on the SDS-PAGE for HA subtype analyzed except for the H1 New Caledonia for which five micrograms of proteins were loaded. a) Expression of H1 from A/New Caledonia/20/1999, b) expression of H1 from A/Brisbane/59/2007, c) expression of H3 from A/Brisbane/10/2007, d) expression of H5 from A/Indonesia/5/2005, and e) expression of B from B/Florida/4/2006. The arrows indicate the immunoband corresponding to HA0; specific *Agrobacterium* strains comprising the specific vectors used for HA expression are indicated at the top of the lanes.

[00153] **FIGURE 89** shows an immunoblot of HA accumulation when co-expressed with Hsp 40 and Hsp70. H1 New Caledonia (AGL1/540) and H3 Brisbane (AGL1/790) were expressed alone or co-expressed with AGL1/R870. HA accumulation level was evaluated by

immunoblot analysis of protein extracts from infiltrated leaves. Whole inactivated virus (WIV) of strain A/New Caledonia/20/99 or Brisbane/10/2007 were used as controls.

DETAILED DESCRIPTION

[00154] 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.

[00155] The following description is of a preferred embodiment.

[00156] 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.

[00157] Furthermore, the present invention provides a method of producing virus like particles (VLPs) in a plant. The method involves introducing a nucleic acid 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.

[00158] 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.

[00159] 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).

[00160] 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.

[00161] 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.

[00162] 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.

[00163] Generally, the term "lipid" refers to a fat-soluble (lipophilic), naturally-occurring molecules. The term is also used more specifically to refer to fatty-acids and their derivatives (including tri-, di-, and monoglycerides and phospholipids), as well as other fat-soluble sterol-containing metabolites or sterols. Phospholipids are a major component of all biological membranes, along with glycolipids, sterols and proteins. Examples of phospholipids include phosphatidylethanolamine, phosphatidylcholine, phosphatidylinositol, phosphatidylserine, phosphatidylglycerol and the like. Examples of sterols include zoosterols (for example, cholesterol) and phytosterols (for example, sitosterol) and steryl-glucoside. Over 200 phytosterols have been identified in various plant species, the most common being campesterol, stigmasterol, ergosterol, brassicasterol, delta-7-stigmasterol, delta-7-avenasterol, daunosterol, sitosterol, 24-methylcholesterol, cholesterol or beta-sitosterol. As one of skill in the art would understand, the lipid composition of the plasma membrane of a cell may vary with the culture or growth conditions of the cell or organism from which the cell is obtained.

[00164] Cell membranes generally comprise lipid bilayers, as well as proteins for various functions. Localized concentrations of particular lipids may be found in the lipid bilayer, referred to as 'lipid rafts'. Without wishing to be bound by theory, lipid rafts may have significant roles in endo and exocytosis, entry or egress of viruses or other infectious agents,

inter-cell signal transduction, interaction with other structural components of the cell or organism, such as intracellular and extracellular matrices.

[00165] With reference to influenza virus, the term “hemagglutinin” or “HA” as used herein refers to a glycoprotein found on the outside of influenza viral particles. HA is a homotrimeric membrane type I glycoprotein, generally comprising a signal peptide, an HA1 domain, and an HA2 domain comprising a membrane-spanning anchor site at the C-terminus and a small cytoplasmic tail (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.

[00166] 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).

[00167] 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.

[00168] 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, H16 or of influenza type B. 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.

[00169] 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 influenza type B (encoded by SEQ ID NO: 26), or a combination thereof. One or more that 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.

[00170] Therefore, the present invention is directed to a VLP comprising one or more than one HA subtype, for example two, three, four, five, six, or more HA subtypes.

[00171] The present invention also provides for nucleic acids encoding hemagglutinins that form VLPs when expressed in plants.

[00172] Exemplary nucleic acids may comprise nucleotide sequences of hemagglutinin from selected strains of influenza subtypes. For example, an A (H1N1) sub-type such as A/New Caledonia/20/99 (H1N1) (SEQ ID NO: 33), the A/Indonesia/5/05 sub-type (H5N1) (comprising construct #660; 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:36), A/Solomon Islands/3/2006 (H1N1; SEQ ID NO:37), A/Singapore/1/57 (H2N2; SEQ ID NO:42), A/Anhui/1/2005 (H5N1; SEQ ID NO:43), A/Vietnam/1194/2004 (H5N1; SEQ ID NO:44), A/Teal/Hong Kong/W312/97 (H6N1; SEQ ID NO:45), A/Hong Kong/1073/99 (H9N2; SEQ ID NO:47), A/Brisbane/10/2007 (H3N2; SEQ ID NO:38), A/Wisconsin/67/2005 (H3N2; SEQ ID NO:39), A/Equine/Prague/56 (H7N7; SEQ ID NO:46), B/Malaysia/2506/2004 (SEQ ID NO:40), or B/Florida/4/2006 (SEQ ID NO:41).

[00173] Correct folding of the hemagglutinins may be important for stability of the protein, formation of multimers, formation of VLPs and function of the HA (ability to hemagglutinate), among other characteristics of influenza hemagglutinins. Folding of a protein may be influenced by one or more factors, including, but not limited to, the sequence of the protein, the relative abundance of the protein, the degree of intracellular crowding, the availability of cofactors that may bind or be transiently associated with the folded, partially folded or unfolded protein, the presence of one or more chaperone proteins, or the like.

[00174] .Heat shock proteins (Hsp) or stress proteins are examples of chaperone proteins, which may participate in various cellular processes including protein synthesis, intracellular trafficking, prevention of misfolding, prevention of protein aggregation, assembly and disassembly of protein complexes, protein folding, and protein disaggregation. Examples of such chaperone proteins include, but are not limited to, Hsp60, Hsp65, Hsp 70, Hsp90, Hsp100, Hsp20-30, Hsp10, Hsp100-200, Hsp100, Hsp90, Lon, TF55, FKBP, cyclophilins, ClpP, GrpE, ubiquitin, calnexin, and protein disulfide isomerases. See, for example, Macario, A.J.L., *Cold Spring Harbor Laboratory Res.* 25:59-70. 1995; Parsell, D.A. & Lindquist, S. *Ann. Rev. Genet.* 27:437-496 (1993); U.S. Patent No. 5,232,833. In some examples, a particular group of chaperone proteins includes Hsp40 and Hsp70.

[00175] Examples of Hsp70 include Hsp72 and Hsc73 from mammalian cells, DnaK from bacteria, particularly mycobacteria such as *Mycobacterium leprae*, *Mycobacterium tuberculosis*, and *Mycobacterium bovis* (such as Bacille-Calmette Guerin: referred to herein as Hsp71). DnaK from *Escherichia coli*, yeast, and other prokaryotes, and BiP and Grp78 from eukaryotes, such as
5 *A. thaliana* (Lin et al. 2001 (Cell Stress and Chaperones 6:201-208). A particular example of an Hsp70 is *A. thaliana* Hsp70 (encoded by SEQ ID NO: 122, or SEQ ID NO: 123). Hsp70 is capable of specifically binding ATP as well as unfolded polypeptides and peptides, thereby participating in protein folding and unfolding as well as in the assembly and disassembly of protein complexes.

10 [00176] Examples of Hsp40 include DnaJ from prokaryotes such as *E. coli* and mycobacteria and HSJ1, HDJ1 and Hsp40 from eukaryotes, such as alfalfa (Frugis et al., 1999. Plant Molecular Biology 40:397-408). A particular example of an Hsp40 is *M. sativa* MsJ1 (encoded by SEQ ID NO: 121, 123 or 114). Hsp40 plays a role as a molecular chaperone in protein folding, thermotolerance and DNA replication, among other cellular activities.

15 [00177] Among Hsps, Hsp70 and its co-chaperone, Hsp40, are involved in the stabilization of translating and newly synthesized polypeptides before the synthesis is complete. Without wishing to be bound by theory, Hsp40 binds to the hydrophobic patches of unfolded (nascent or newly transferred) polypeptides, thus facilitating the interaction of Hsp70-ATP complex with the polypeptide. ATP hydrolysis leads to the formation of a stable complex
20 between the polypeptide, Hsp70 and ADP, and release of Hsp40. The association of Hsp70-ADP complex with the hydrophobic patches of the polypeptide prevents their interaction with other hydrophobic patches, preventing the incorrect folding and the formation of aggregates with other proteins (reviewed in Hartl, FU. 1996. Nature 381:571-579).

[00178] Again, without wishing to be bound by theory, as protein production increases in
25 a recombinant protein expression system, the effects of crowding on recombinant protein expression may result in aggregation and/or reduced accumulation of the recombinant protein resulting from degradation of misfolded polypeptide. Native chaperone proteins may be able to facilitate correct folding of low levels of recombinant protein, but as the expression levels increase, native chaperones may become a limiting factor. High levels of expression of
30 hemagglutinin in the agroinfiltrated leaves may lead to the accumulation of hemagglutinin polypeptides in the cytosol, and co-expression of one or more than one chaperone proteins such as Hsp70, Hsp40 or both Hsp70 and Hsp40 may increase stability in the cytosol of the cells

expressing the polypeptides cells, thus reducing the level of misfolded or aggregated hemagglutinin polypeptides, and increasing the number of polypeptides accumulate as stable hemagglutinin, exhibiting tertiary and quaternary structural characteristics that allow for hemagglutination and/or formation of virus-like particles.

5 [00179] Therefore, the present invention also provides for a method of producing influenza VLPs in a plant, wherein a first nucleic acid encoding an influenza HA is co-expressed with a second nucleic acid encoding a chaperone. The first and second nucleic acids may be introduced to the plant in the same step, or may be introduced to the plant sequentially. The present invention also provides for a method of producing influenza VLPs in a plant, where the
10 plant comprises the first nucleic acid, and the second nucleic acid is subsequently introduced.

[00180] The present invention also provides for a plant comprising a nucleic acid encoding one, or more than one influenza hemagglutinin and a nucleic acid encoding one or more than one chaperones.

[00181] Processing of an N-terminal signal peptide (SP) sequence during expression
15 and/or secretion of influenza hemagglutinins has been proposed to have a role in the folding process. The term "signal peptide" refers generally to a short (about 5-30 amino acids) sequence of amino acids, found generally at the N-terminus of a hemagglutinin polypeptide that may direct translocation of the newly-translated polypeptide to a particular organelle, or aid in positioning of specific domains of the polypeptide. The signal peptide of hemagglutinins target the
20 translocation of the protein into the endoplasmic reticulum and have been proposed to aid in positioning of the N-terminus proximal domain relative to a membrane-anchor domain of the nascent hemagglutinin polypeptide to aid in cleavage and folding of the mature hemagglutinin. Removal of a signal peptide (for example, by a signal peptidase), may require precise cleavage and removal of the signal peptide to provide the mature hemagglutinin – this precise cleavage
25 may be dependent on any of several factors, including a portion or all of the signal peptide, amino acid sequence flanking the cleavage site, the length of the signal peptide, or a combination of these, and not all factors may apply to any given sequence.

[00182] A signal peptide may be native to the hemagglutinin being expressed, or a recombinant hemagglutinin comprising a signal peptide from a first influenza type, subtype or
30 strain with the balance of the hemagglutinin from a second influenza type, subtype or strain. For

example the native SP of HA subtypes H1, H2, H3, H5, H6, H7, H9 or influenza type B may be used to express the HA in a plant system.

[00183] A signal peptide may also be non-native, for example, from a structural protein or hemagglutinin of a virus other than influenza, or from a plant, animal or bacterial polypeptide.

- 5 An exemplary signal peptide is that of alfalfa protein disulfide isomerase (PDISP) (nucleotides 32-103 of Accession No. Z11499; SEQ ID NO: 34; Figure 17; amino acid sequence MAKNV AIFGLLFSLLLLVPSQIFAE).

[00184] The present invention also provides for an influenza hemagglutinin comprising a native, or a non-native signal peptide, and nucleic acids encoding such hemagglutinins.

- 10 [00185] 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-
15 chemical properties are provided in Table 1.

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	HA0 ¹	HA1	HA1 ¹	HA2	HA2 ¹	HA0	HA1	HA2
774	H1	A/Brisbane/59/2007	548		222	9		2	61	75			25	28	6.4		5.3
775	H1	A/Solomon Islands/3/2006	548		222	9		2	61	75			25	28	6.1		5.3
776	H3	A/Brisbane/10/2007	550		221	12		1	62	80		54	25	27	8.5		5.2
777	H3	A/Wisconsin/67/2005	550		221	11		1	62	79		52	25	27	8.8		5.3
778	B	B/Malaysia/2506/2004	570		223	12		4	62	80			24	30	8.0		4.5
779	B	B/Florida/4/2006	569		223	10		3	62	77			24	29	8.0		4.5
780	H2	A/Singapore/1/57	547		222	6		2	62	71			25	28	6.0		4.9
781	H5	A/Anhui/1/2005	551		222	7		2	62	73			25	28	6.2		4.7
782	H5	A/Vietnam/1194/2004	552		222	7		2	63	74			25	28	6.4		4.8
783	H6	A/Teal/Hong Kong/W312/97	550		222	8		3	62	75		45	25	30	5.7		5.6
784	H7	A/Equine/Prague/56	552		221	6		2	62	71		43	25	28	8.9		4.9
785	H9	A/Hong Kong/1073/99	542		199	9		2	61	75		46	23	26	8.4		5.3

[00186] 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. The present invention also includes a nucleotide sequence that hybridizes under stringent hybridisation conditions to SEQ ID NO:28; SEQ ID NO:3; SEQ ID NO:11. The present invention also includes a nucleotide sequence that hybridizes under stringent hybridisation conditions to a complement of SEQ ID NO:28; SEQ ID NO:3; SEQ ID NO:1. These nucleotide sequences that hybridize to SEQ ID or a complement of SEQ ID encode a hemagglutinin protein that, when expressed forms a VLP, and the VLP induces 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.

[00187] The present invention also includes nucleotide sequences SEQ ID NO:12 SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 23, SEQ ID NO: 24, SEQ ID NO: 25, SEQ ID NO: 26, SEQ ID NO:27, SEQ ID NO: 33, SEQ ID NO: 35, SEQ ID NO: 36, SEQ ID NO:37, SEQ ID NO:38, SEQ ID NO:39, SEQ ID NO:40, SEQ ID NO:41, SEQ ID NO:42, SEQ ID NO:43, SEQ ID NO:44, SEQ ID NO:45, SEQ ID NO:46 or SEQ ID NO:47. The present invention also includes a nucleotide sequence that hybridizes under stringent hybridisation conditions to SEQ ID NO:12 SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 23, SEQ ID NO: 24, SEQ ID NO: 25, SEQ ID NO: 26, SEQ ID NO:27, SEQ ID NO: 33, SEQ ID NO: 35, SEQ ID NO: 36, SEQ ID NO:37, SEQ ID NO:38, SEQ ID NO:39, SEQ ID NO:40, SEQ ID NO:41, SEQ ID NO:42, SEQ ID NO:43, SEQ ID NO:44, SEQ ID NO:45, SEQ ID NO:46 or SEQ ID NO:47. The present invention also includes a nucleotide sequence that hybridizes under stringent hybridisation conditions to a complement of SEQ ID NO:12 SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 23, SEQ ID NO: 24, SEQ ID NO: 25, SEQ ID NO: 26, SEQ ID NO:27, SEQ ID NO: 33, SEQ ID NO: 35, SEQ ID NO: 36, SEQ ID NO:37, SEQ ID NO:38, SEQ ID NO:39, SEQ ID NO:40, SEQ ID NO:41, SEQ ID NO:42, SEQ ID NO:43, SEQ ID NO:44, SEQ ID NO:45, SEQ ID NO:46 or SEQ ID NO:47. These nucleotide sequences that hybridize to SEQ ID NO:12 SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16,

SEQ ID NO: 17, SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 23, SEQ ID NO: 24, SEQ ID NO: 25, SEQ ID NO: 26, SEQ ID NO: 27, SEQ ID NO: 33, SEQ ID NO: 35, SEQ ID NO: 36, SEQ ID NO: 37, SEQ ID NO: 38, SEQ ID NO: 39, SEQ ID NO: 40, SEQ ID NO: 41, SEQ ID NO: 42, SEQ ID NO: 43, SEQ ID NO: 44, SEQ ID NO: 45, SEQ ID NO: 46 or SEQ ID NO: 47 or a complement of SEQ ID NO: 12 SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 23, SEQ ID NO: 24, SEQ ID NO: 25, SEQ ID NO: 26, SEQ ID NO: 27, SEQ ID NO: 33, SEQ ID NO: 35, SEQ ID NO: 36, SEQ ID NO: 37, SEQ ID NO: 38, SEQ ID NO: 39, SEQ ID NO: 40, SEQ ID NO: 41, SEQ ID NO: 42, SEQ ID NO: 43, SEQ ID NO: 44, SEQ ID NO: 45, SEQ ID NO: 46 or SEQ ID NO: 47 encode a hemagglutinin protein that, when expressed forms a VLP, and the VLP induces 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.

[00188] In some embodiments, the present invention also includes nucleotide sequences SEQ ID NO: 33, SEQ ID NO: 35, SEQ ID NO: 36, SEQ ID NO: 37, SEQ ID NO: 38, SEQ ID NO: 39, SEQ ID NO: 40, SEQ ID NO: 41, SEQ ID NO: 42, SEQ ID NO: 43, SEQ ID NO: 44, SEQ ID NO: 45, SEQ ID NO: 46 or SEQ ID NO: 47, encoding HA from H1, H2, H3, H5, H7 or H9 subtypes of influenza A, or HA from type B influenza. The present invention also includes a nucleotide sequence that hybridizes under stringent hybridisation conditions to SEQ ID NO: 33, SEQ ID NO: 35, SEQ ID NO: 36, SEQ ID NO: 37, SEQ ID NO: 38, SEQ ID NO: 39, SEQ ID NO: 40, SEQ ID NO: 41, SEQ ID NO: 42, SEQ ID NO: 43, SEQ ID NO: 44, SEQ ID NO: 45, SEQ ID NO: 46 or SEQ ID NO: 47. The present invention also includes a nucleotide sequence that hybridizes under stringent hybridisation conditions to a complement of SEQ ID NO: 33, SEQ ID NO: 35, SEQ ID NO: 36, SEQ ID NO: 37, SEQ ID NO: 38, SEQ ID NO: 39, SEQ ID NO: 40, SEQ ID NO: 41, SEQ ID NO: 42, SEQ ID NO: 43, SEQ ID NO: 44, SEQ ID NO: 45, SEQ ID NO: 46 or SEQ ID NO: 47. These nucleotide sequences that hybridize to SEQ ID NO: 33, SEQ ID NO: 35, SEQ ID NO: 36, SEQ ID NO: 37, SEQ ID NO: 38, SEQ ID NO: 39, SEQ ID NO: 40, SEQ ID NO: 41, SEQ ID NO: 42, SEQ ID NO: 43, SEQ ID NO: 44, SEQ ID NO: 45, SEQ ID NO: 46 or SEQ ID NO: 47 or a complement of SEQ ID NO: 33, SEQ ID NO: 35, SEQ ID NO: 36, SEQ ID NO: 37, SEQ ID NO: 38, SEQ ID NO: 39, SEQ ID NO: 40, SEQ ID NO: 41, SEQ ID NO: 42, SEQ

ID NO:43, SEQ ID NO:44, SEQ ID NO:45, SEQ ID NO:46 or SEQ ID NO:47 encode a hemagglutinin protein that, when expressed forms a VLP, and the VLP induces 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.

[00189] 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.

[00190] 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 administered to a subject, induces an immune response.

[00191] Additionally, the present invention includes nucleotide sequences that are characterized as having about 70, 75, 80, 85, 87, 90, 91, 92, 93 94, 95, 96, 97, 98, 99, 100% or any amount therebetween, sequence identity, or sequence similarity, with the nucleotide sequence of SEQ ID NO:12 SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16,

SEQ ID NO: 17, SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 23, SEQ ID NO: 24, SEQ ID NO: 25, SEQ ID NO: 26, SEQ ID NO: 27, SEQ ID NO: 33, SEQ ID NO: 35, SEQ ID NO: 36, SEQ ID NO: 37, SEQ ID NO: 38, SEQ ID NO: 39, SEQ ID NO: 40, SEQ ID NO: 41, SEQ ID NO: 42, SEQ ID NO: 43, SEQ ID NO: 44, SEQ ID NO: 45, SEQ ID NO: 46 or SEQ ID NO: 47, 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 administered to a subject, induces an immune response.

[00192] Additionally, the present invention includes nucleotide sequences that are characterized as having about 70, 75, 80, 85, 87, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100% or any amount therebetween, sequence identity, or sequence similarity, with the nucleotide sequence of SEQ ID NO: 33, SEQ ID NO: 35, SEQ ID NO: 36, SEQ ID NO: 37, SEQ ID NO: 38, SEQ ID NO: 39, SEQ ID NO: 40, SEQ ID NO: 41, SEQ ID NO: 42, SEQ ID NO: 43, SEQ ID NO: 44, SEQ ID NO: 45, SEQ ID NO: 46 or SEQ ID NO: 47, 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 administered to a subject, induces an immune response.

[00193] 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), or influenza type B (encoded by SEQ ID NO: 26); see Figures 10A to 100), and nucleotide sequences that are characterized as having from about 70 to 100% or any amount therebetween, 80 to 100% or any amount there between, 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 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 immune response.

10 [00194] An "immune response" generally refers to a response of the adaptive immune system. The adaptive immune system generally comprises a humoral response, and a cell-mediated response. The humoral response is the aspect of immunity that is mediated by secreted antibodies, produced in the cells of the B lymphocyte lineage (B cell). Secreted antibodies bind to antigens on the surfaces of invading microbes (such as viruses or bacteria), which flags them for destruction. Humoral immunity is used generally to refer to antibody production and the processes that accompany it, as well as the effector functions of antibodies, including Th2 cell activation and cytokine production, memory cell generation, opsonin promotion of phagocytosis, pathogen elimination and the like. The terms "modulate" or "modulation" or the like refer to an increase or decrease in a particular response or parameter, as determined by any of several assays generally known or used, some of which are exemplified herein.

[00195] 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.

[00196] 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.

[00197] 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).

- 5 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.

- [00198] 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
10 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.

- [00199] 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
15 using by ELISA (e.g. BD Biosciences OptEIA kits). Peripheral blood mononuclear cells (PBMC) or splenocytes obtained from a subject may be cultured, and the supernatant analyzed. T lymphocytes may also be quantified by fluorescence-activated cell sorting (FACS), using marker specific fluorescent labels and methods as are known in the art.

- [00200] A microneutralization assay may also be conducted to characterize an immune
20 response in a subject, see for example the methods of Rowe et al., 1973. Virus neutralization titers may be obtained several ways, including: 1) enumeration of lysis plaques (plaque assay) following crystal violet fixation/coloration of cells; 2) microscopic observation of cell lysis in culture; 3) ELISA and spectrophotometric detection of NP virus protein (correlate with virus infection of host cells)

- 25 [00201] Sequence identity or sequence similarity may be determined using a nucleotide sequence comparison program, such as that provided within DNASIS (for example, using, but not limited to, the following parameters: GAP penalty 5, #of top diagonals 5, fixed GAP penalty 10, k-tuple 2, floating gap 10, and window size 5). However, other methods of alignment of sequences for comparison are well-known in the art for example the algorithms of Smith &
30 Waterman (1981, Adv. Appl. Math. 2:482), Needleman & Wunsch (J. Mol. Biol. 48:443, 1970), Pearson & Lipman (1988, Proc. Nat'l. Acad. Sci. USA 85:2444), and by computerized

implementations of these algorithms (e.g. GAP, BESTFIT, FASTA, and BLAST), or by manual alignment and visual inspection.

[00202] The term "hemagglutinin domain" refers to a peptide comprising either the HA0 domain, or the HA1 and HA2 domains (alternately referred to as HA1 and HA2 fragments).

- 5 HA0 is a precursor of the HA1 and HA2 fragments. The HA monomer may be generally subdivided in 2 functional domains - the stem domain and the globular head, or head domain. The stem domain is involved in infectivity and pathogenicity of the virus via the conformational change it may undergo when exposed to acidic pH. The stem domain may be further subdivided into 4 subdomains or fragments - the fusion sub-domain or peptide (a hydrophobic stretch of amino acids involved in fusion with the host membrane in the acidic pH
- 10 conformational state); the stem sub-domain (may accommodate the two or more conformations), the transmembrane domain or sub-domain (TmD) (involved in the affinity of the HA for lipid rafts), and the cytoplasmic tail (cytoplasmic tail sub-domain) (Ctail) (involved in secretion of HA). The globular head is divided in 2 subdomains, the RB subdomain and the vestigial esterase
- 15 domain (E). The E subdomain may be partially or fully buried and not exposed at the surface of the globular head, thus some antibodies raised against HA bind to the RB subdomain.

[00203] 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
- 20 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
- 25 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.

- [00204] The VLPs produced from influenza derived proteins, in accordance with the
- 30 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.

[00205] 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 a plant cell, an insect cell, fungi, and other organisms including sponge, coelenterata, annelida, arthropoda, mollusca, nemathelminthea, trochelmintes, plathelminthes, chaetognatha, tentaculate, chlamydia, spirochetes, gram-positive bacteria, cyanobacteria, archaebacteria, or the like. See, for example Glycoforum (URL: glycoforum.gr.jp/science/word/evolution/ES-A03E.html) or Gupta et al., 1999. Nucleic Acids Research 27:370-372; or Toukach et al., 2007. Nucleic Acids Research 35:D280-D286; or URL:glycostructures.jp (Nakahara et al., 2008. Nucleic Acids Research 36:D368-D371; published online October 11, 2007 doi:10.1093/NAR/gkm833). 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.

[00206] 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'. 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.

[00207] VLPs may be assessed for structure and size by, for example, hemagglutination assay, electron microscopy, or by size exclusion chromatography.

[00208] 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 a calibration standard. Following chromatography, fractions may be further analyzed by immunoblot to determine the protein complement of the fraction.

[00209] 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 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 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	++	+

[00210] A fragment or portion of a protein, fusion protein or polypeptide includes a peptide or polypeptide comprising a subset of the amino acid complement of a particular protein or polypeptide, provided that the fragment can form a VLP when expressed. The fragment may, for example, comprise an antigenic region, a stress-response-inducing region, or a region comprising a functional domain of the protein or polypeptide. The fragment may also comprise a region or domain common to proteins of the same general family, or the fragment may include sufficient amino acid sequence to specifically identify the full-length protein from which it is derived.

[00211] 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.

[00212] 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.

[00213] 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.

[00214] 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.

[00215] 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.

[00216] 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.

[00217] 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.

[00218] However, other envelope proteins may be used within the methods of the present invention as would be know to one of skill in the art.

[00219] 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.

[00220] 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 immunogenic homotypic macromolecular protein structures, including subviral influenza particles and influenza VLP, in transformed plant cells or transformed insect cells.

[00221] 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 (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, 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.

[00222] 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 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), 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 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 (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.

[00223] The resulting cDNA copies of these genes may be cloned in a suitable 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.

[00224] The present invention is further directed to a gene construct 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 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. A regulatory element or regulatory region may enhance translation of a nucleotide sequence to which is it operatively linked – the nucleotide sequence may encode a protein or polypeptide. Another example of a regulatory region is that derived from the untranslated regions of the Cowpea Mosaic Virus (CPMV), which may be used to preferentially translate the nucleotide sequence to which it is operatively linked. This CPMV regulatory region comprises a CMPV-HT system - see, for example, Sainsbury et al, 2008, Plant Physiology 148: 1212-1218.

[00225] 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.

[00226] 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), 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 plastocyanin regulatory element and an influenza HA are exemplified herein by SEQ ID NOs: 36-47.

5 [00227] 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 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
10 influenza hemagglutinins obtained from new strains as additional mutations continue to occur. Due to the known sequence variability between different influenza hemagglutinins, the present 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.

[00228] Sequence alignments and consensus sequences may be determined using any of
15 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.

[00229] 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
20 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; 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
25 homotrimer with each monomer comprising a receptor binding domain (HA1) and a membrane-anchoring domain (HA2) linked by a single 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
30 typically conserved in length.

- 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 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 (Berman et al. 2003. Nature Structural Biology 10:980; URL: 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.

[00230] 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 encoding a hemagglutinin amino acid sequence, that forms VLPs in a plant, and includes known sequences and variant sequences that may develop.

[00231] 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:

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 (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.

[00232] 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.

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

SEQ ID NO.		Sequence				
10	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
	76					
Consensus		mkxkllvllc	tftatyadti	cigyhannst	dtvdtvlekn	vtvthsvnll
20	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
	76					
Consensus		exshngklcl	lkgiaplqlg	ncsvagwilg	npecellis.	eswsyive.p
30	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
	76					
Consensus		npengtcypg	xfadyeelre	qlssvssfer	feifpkessw	pnhtxtgvsa
35	151					200
	75	SCSHNGKSSF	YRNLLWLTGK	NGLYPNLSKS	YVNNKEKEVL	VLWG VHPPN
	9	SCSHNGKSSF	YRNLLWLTGK	NGLYPNLSKS	YVNNKEKEVL	VLWG VHPPN
	48	SCSHNGESSF	YRNLLWLTGK	NGLYPNLSKS	YANNKEKEVL	VLWG VHPPN
	49	SCSHNGESSF	YKNLLWLTGK	NGLYPNLSKS	YANNKEKEVL	VLWG VHPPN
	76					
Consensus		scshngxssf	yxnllwltgk	nglypnlsks	yxnnkekevl	vlwgvhppn
40	201					250
	75	IGNQRALYHT	ENAYVSVVSS	HYSRRFTPEI	AKRPKVRDQE	GRINYYWTLL
	9	IGNQRALYHT	ENAYVSVVSS	HYSRRFTPEI	AKRPKVRDQE	GRINYYWTLL
	48	IGDQKALYHT	ENAYVSVVSS	HYSRKFTPEI	AKRPKVRDQE	GRINYYWTLL
	49	IGDQRALYHK	ENAYVSVVSS	HYSRKFTPEI	AKRPKVRDQE	GRINYYWTLL
	76		MSLLT	EVETYVLSII	PSGPLKAEIA	QRLEDVFAGK
Consensus		igxqxalyhx	enayvsvvss	hysrxftpei	akrPkvr#qe	gRi#yywtll
50	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
	76	NTDLEVLMEW	...LKTRPIL	SPLTKGILGF	VFTLTVPSE	GLQRRRFVQN
Consensus		#pgdt!ifEa	ngnLiapxya	faLsrGfgsg	!itsnaPm#x	cdakcqtPQg

		301			350
	75	AINSSLPFQN	VHPVTIGIECP	KYVRS AKLRM	VT.GLRNIPS IQSRGLFGAI
5	9	AINSSLPFQN	VHPVTIGIECP	KYVRS AKLRM	VT.GLRNIPS IQSRGLFGAI
	48	AINSSLPFQN	VHPVTIGIECP	KYVRS AKLRM	VT.GLRNIPS IQSRGLFGAI
	49	AINSSLPFQN	VHPVTIGIECP	KYVRS AKLRM	VT.GLRNIPS IQSRGLFGAI
	76	ALNG.....N	GDPNNMDKAV	KLYRKLKREI	TFHGAKEISL SYSAGALASC
	Consensus	AiNsslpfqN	vhPvtigecp	KyvRsaKlrm	vtxGlr#Ips iqSrGlfgai
10		351			400
	75	AGFIEGGWTG	MVDGWYGYHH	QNEQSGSYAA	DQKSTQNAIN GITNKNVSVI
	9	AGFIEGGWTG	MVDGWYGYHH	QNEQSGSYAA	DQKSTQNAIN GITNKNVSVI
	48	AGFIEGGWTG	MVDGWYGYHH	QNEQSGSYAA	DQKSTQNAIN GITNKNVSVI
	49	AGFIEGGWTG	MVDGWYGYHH	QNEQSGSYAA	DQKSTQNAIN GITNKNVSVI
15	76	MGLIYNRM.G	AVTTEVAFGL	VCATCEQIAD	SQHRSHRQMV TTTNPLIRHE
	Consensus	aGfIeggwtG	mVdgwyg%hh	qneqsgsyAa	dQkstqnain giTNkvnsvi
		401			450
20	75	EKMNTQFTAV	GKEFNKLERR	MENLNKKVDD	GFLDIWTYNA ELLVLENER
	9	EKMNTQFTAV	GKEFNKLERR	MENLNKKVDD	GFLDIWTYNA ELLVLENER
	48	EKMNTQFTAV	GKEFNKLERR	MENLNKKVDD	GFIDIWTYNA ELLVLENER
	49	EKMNTQFTAV	GKEFNKLERR	MENLNKKVDD	GFIDIWTYNA ELLVLENER
	76	NRMVLASTTA	.KAMEQMAGS	SEQAAEAMEV	A.....S QARQMVQAMR
25	Consensus	#kMntqfTav	gKef#k\$err	mE#lnkkv#d	gfxdiwtyna #llv\$l#neR
		451			500
	75	TLDFHDSNVK	NLYEKVKS QL	KNNAKEIGNG	CFEFYHKCNN ECMESVKNGT
	9	TLDFHDSNVK	NLYEKVKS QL	KNNAKEIGNG	CFEFYHKCNN ECMESVKNGT
30	48	TLDFHDSNVK	NLYEKVKS QL	KNNAKEIGNG	CFEFYHKCND ECMESVKNGT
	49	TLDFHDSNVK	NLYEKVKS QL	KNNAKEIGNG	CFEFYHKCND ECMESVKNGT
	76	TIGTHPSSSA	GLKNDLLENL	QAYQKRMGVQ	MQRFK.....
	Consensus	TldfHdSnvk	nLy#kvks#L	knnaKeiGng	cfeFyhkcnx ecmesvkngt
		501			550
35	75	YDYPKYSEES	KLNREKIDGV	KLESMGVYQI	LAIYSTVASS LVLLVSLGAI
	9	YDYPKYSEES	KLNREKIDGV	KLESMGVYQI	LAIYSTVASS LVLLVSLGAI
	48	YDYPKYSEES	KLNREKIDGV	KLESMGVYQI	LAIYSTVASS LVLLVSLGAI
	49	YDYPKYSEES	KLNREKIDGV	KLESMGVYQI	LAIYSTVASS LVLLVSLGAI
40	76				
	Consensus	ydyppysees	klntrekidgv	klesmgvyqi	laiystvass lvllvslgai
		551		566	
	75	SFWMCSNGSL	QCRICI		
45	9	SFWMCSNGSL	QCRICI		
	48	SFWMCSNGSL	QCRICI		
	49	SFWMCSNGSL	QCRICI		
	76				
	Consensus	sfwmcsgsl	qcrici		

50 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.

- 5 [00233] As another example of such variation, a sequence alignment and consensus sequence for HA of A/Anhui/1/2005 (H5N1) (SEQ ID NO: 55), 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	MEKIVLLFAI	VSLVKSDQIC	IGYHANNSTE	QVDTIMEKNV	TVTHAQDILE
	55	MEKIVLLLLAI	VSLVKSDQIC	IGYHANNSTE	QVDTIMEKNV	TVTHAQDILE
15	Consensus	MEKIVLLLLAI	VSLVKSDQIC	IGYHANNSTE	QVDTIMEKNV	TVTHAQDILE
	51					100
	10	KTHNGKLCDL	DGVKPLILRD	CSVAGWLLGN	PMCDEFINVP	EWSYIVEKAN
	56	KTHNGKLCDL	DGVKPLILRD	CSVAGWLLGN	PMCDEFINVP	EWSYIVEKAN
	55	KTHNGKLCDL	DGVKPLILRD	CSVAGWLLGN	PMCDEFINVP	EWSYIVEKAN
20	Consensus	KTHNGKLCDL	DGVKPLILRD	CSVAGWLLGN	PMCDEFINVP	EWSYIVEKAN
	101					150
	10	PTNDLCYPGS	FNDYEELKHL	LSRINHFEDI	QIIPKSSWSD	HEASSGVSSA
	56	PVNDLCYPGD	FNDYEELKHL	LSRINHFEDI	QIIPKSSWSS	HEASLGVSSA
25	55	PANDLCYPGN	FNDYEELKHL	LSRINHFEDI	QIIPKSSWSD	HEASSGVSSA
	Consensus	PxNDLCYPGx	FNDYEELKHL	LSRINHFEDI	QIIPKSSWsd	HEAssGVSSA
	151					200
	10	CPYLGSPSFF	RNVVWLIKKN	STYPTIKKSY	NNTNQEDLLV	LWGIHHPNDA
30	56	CPYQGKSSFF	RNVVWLIKKN	STYPTIKRSY	NNTNQEDLLV	LWGIHHPNDA
	55	CPYQGTPSFF	RNVVWLIKKN	NTYPTIKRSY	NNTNQEDLLI	LWGIHHSNDA
	Consensus	CPYqGxpSFF	RNVVWLIKKN	sTYPTIKrSY	NNTNQEDLL!	LWGIHHpNDA
	201					250
35	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
40	251					300
	10	PNDAINFESN	GNFIAPEYAY	KIVKKGDSAI	MKSELEYGNC	NTKCQTPMGA
	56	PNDAINFESN	GNFIAPEYAY	KIVKKGDSTI	MKSELEYGNC	NTKCQTPMGA
	55	PNDAINFESN	GNFIAPEYAY	KIVKKGDSAI	VKSEVEYGNC	NTKCQTPIGA
45	Consensus	PNDAINFESN	GNFIAPEYAY	KIVKKGDSaI	mKSELEYGNC	NTKCQTPmGA
	301					350
	10	INSSMPFHNI	HPLTIGECPK	YVKSNNRLVLA	TGLRNSPQRE	SRRKKRGLFG
	56	INSSMPFHNI	HPLTIGECPK	YVKSNNRLVLA	TGLRNSPQRE	RRRKKRGLFG
	55	INSSMPFHNI	HPLTIGECPK	YVKSNNKLVA	TGLRNSPLRE	RRR.K.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
20	Consensus	ERTLDFHDSN	VKNLYDKVRL	QLRDNAKELG	NGCFEFYHKC	DNECMES!RN
		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
 35 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.

40 [00234] 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.

[00235] 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,
 45 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),

5 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),

10 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.

[00236] Further, an amino acid sequence encoded by a nucleic acid may be easily determined, as the codon or codons for each amino acid are known. Provision of a nucleic acid,

15 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,

20 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

25 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)).

[00237] In plants, influenza VLPs bud from the plasma membrane (see Example 5, and

30 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. Plant lipids can stimulate specific immune cells

and enhance the immune response induced. Plant membranes are made of lipids, phosphatidylcholine (PC) and phosphatidylethanolamine (PE), and also contain glycosphingolipids, saponins, and phytosterols. Additionally, lipid rafts are also found in plant plasma membranes - these microdomains are enriched in sphingolipids and sterols. In plants, a variety of phytosterols are known to occur, including stigmasterol, sitosterol, 24-methylcholesterol and cholesterol (Mongrand et al., 2004).

[00238] PC and PE, as well as glycosphingolipids can bind to CD1 molecules expressed by mammalian immune cells such as antigen-presenting cells (APCs) like dendritic cells and macrophages and other cells including B and T lymphocytes in the thymus and liver (Tsuji M., 2006). CD1 molecules are structurally similar to major histocompatibility complex (MHC) molecules of class I and their role is to present glycolipid antigens to NKT cells (Natural Killer T cells). Upon activation, NKT cells activate innate immune cells such as NK cells and dendritic cells and also activate adaptive immune cells like the antibody-producing B cells and T-cells.

[00239] 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.

[00240] The phytosterols present in an influenza VLP complexed with a lipid bilayer, such as an plasma-membrane derived envelope may provide for an advantageous vaccine composition. Without wishing to be bound by theory, plant-made VLPs complexed with a lipid bilayer, such as a plasma-membrane derived envelope, may induce a stronger immune reaction than VLPs made in other expression systems, and may be similar to the immune reaction induced by live or attenuated whole virus vaccines.

[00241] 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.

[00242] The VLP produced within a plant may include an HA comprising plant-specific N-glycans. Therefore, this invention also provides for a VLP comprising HA having plant specific N-glycans.

[00243] 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
5 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 [00244] 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
15 tail, transmembrane domain, stem region) of N-acetylglucosaminyl transferase (GNT1), to produce a GNT1-GalT hybrid enzyme, and the hybrid enzyme may be co-expressed with HA. The HA may also be co-expressed along with a nucleotide sequence encoding N-acetylglucosaminyltransferase III (GnT-III), for example but not limited to mammalian GnT-III or human GnT-III, GnT-III from other sources may also be used. Additionally, a GNT1-GnT-III
20 hybrid enzyme, comprising the CTS of GNT1 fused to GnT-III may also be used .

[00245] Therefore the present invention also includes VLP's comprising HA having modified N-glycans.

[00246] 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.
25 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.

[00247] By "regulatory region", "regulatory element" or "promoter" it is meant a portion
30 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.

[00248] 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.

[00249] There are several types of regulatory regions, including those that are developmentally regulated, inducible or constitutive. A regulatory region that is developmentally regulated, or controls the differential expression of a gene under its control, is activated within certain organs or tissues of an organ at specific times during the development of that organ or tissue. However, some regulatory regions that are developmentally regulated may preferentially be active within certain organs or tissues at specific developmental stages, they may also be active in a developmentally regulated manner, or at a basal level in other organs or tissues within the plant as well. Examples of tissue-specific regulatory regions, for example see-

specific a regulatory region, include the napin promoter, and the cruciferin promoter (Rask et al., 1998, *J. Plant Physiol.* 152: 595-599; Bilodeau et al., 1994, *Plant Cell* 14: 125-130). An example of a leaf-specific promoter includes the plastocyanin promoter (Figure 1b or SEQ ID NO:23); US 7,125,978, which is incorporated herein by reference).

5 [00250] An inducible regulatory region is one that is capable of directly or indirectly activating transcription of one or more DNA sequences or genes in response to an inducer. In the absence of an inducer the DNA sequences or genes will not be transcribed. Typically the protein factor that binds specifically to an inducible regulatory region to activate transcription may be present in an inactive form, which is then directly or indirectly converted to the active
10 form by the inducer. However, the protein factor may also be absent. The inducer can be a chemical agent such as a protein, metabolite, growth regulator, herbicide or phenolic compound or a physiological stress imposed directly by heat, cold, salt, or toxic elements or indirectly through the action of a pathogen or disease agent such as a virus. A plant cell containing an inducible regulatory region may be exposed to an inducer by externally applying the inducer to
15 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),
20 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
25 incorporated by reference) and the auxin inducible element, DR5 (Ulmasov, T., et al., 1997, *Plant Cell* 9, 1963-1971; which is incorporated by reference).

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.,
30 1985, *Nature*, 313: 810-812), the rice actin 1 (Zhang et al, 1991, *Plant Cell*, 3: 1155-1165), actin 2 (An et al., 1996, *Plant J.*, 10: 107-121), or tms 2 (U.S. 5,428,147, which is incorporated herein by reference), and triosephosphate isomerase 1 (Xu et. al., 1994, *Plant Physiol.* 106: 459-467)

genes, the maize ubiquitin 1 gene (Cornejo et al, 1993, Plant Mol. Biol. 29: 637-646), the *Arabidopsis* ubiquitin 1 and 6 genes (Holtorf et al, 1995, Plant Mol. Biol. 29: 637-646), and the tobacco translational initiation factor 4A gene (Mandel et al, 1995 Plant Mol. Biol. 29: 995-1004). The term "constitutive" as used herein does not necessarily indicate that a gene under control of the constitutive regulatory region is expressed at the same level in all cell types, but that the gene is expressed in a wide range of cell types even though variation in abundance is often observed. Constitutive regulatory elements may be coupled with other sequences to further enhance the transcription and/or translation of the nucleotide sequence to which they are operatively linked. For example, the CMPV-HT system (Sainsbury et al, 2008, Plant Physiology 148: 1212-1218) is derived from the untranslated regions of the Cowpea mosaic virus (COMV) and demonstrates enhanced translation of the associated coding sequence.

[00251] By "native" it is meant that the nucleic acid or amino acid sequence is naturally occurring, or "wild type".

[00252] 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.

[00253] 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.

[00254] 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 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 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.

5 [00255] Non-limiting examples of suitable 3' regions are the 3' transcribed non-translated regions containing a polyadenylation signal of *Agrobacterium* tumor inducing (Ti) plasmid genes, such as the nopaline synthase (Nos gene) 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
10 plastocyanin expression (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)

[00256] As described herein, promoters comprising enhancer sequences with demonstrated efficiency in leaf expression, have been found to be effective in transient
15 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.

[00257] To aid in identification of transformed plant cells, the constructs of this invention
20 may be further manipulated to include plant selectable markers. Useful selectable markers include enzymes that provide for resistance to chemicals such as an antibiotic for example, gentamycin, hygromycin, kanamycin, or herbicides such as phosphinothrycin, glyphosate, chlorosulfuron, and the like. Similarly, enzymes providing for production of a compound identifiable by colour change such as GUS (beta-glucuronidase), or luminescence, such as
25 luciferase or GFP, may be used.

[00258] 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
30 selectable markers are used to facilitate identification of transformed plant cells. Once callus forms, shoot formation can be encouraged by employing the appropriate plant hormones in

accordance with known methods and the shoots transferred to rooting medium for regeneration of plants. The plants may then be used to establish repetitive generations, either from seeds or using vegetative propagation techniques. Transgenic plants can also be generated without using tissue cultures.

5 [00259] 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 HA0 for VLP production, in accordance with the present invention.

[00260] 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, 10 both monocots and dicots, for example but not limited to corn, cereal plants, wheat, barley, oat, *Nicotiana* spp, *Brassica* spp, soybean, bean, pea, alfalfa, potato, tomato, ginseng, and *Arabidopsis*.

[00261] Methods for stable transformation, and regeneration of these organisms are 15 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.

[00262] 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 20 and the transfer of genetic information considered stable, or the transfer may be transient and the transfer of genetic information is not inheritable.

[00263] 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 25 extracts of plants, or a combination thereof. Further, plant matter may comprise plants, plant cells, tissue, a liquid extract, or a combination thereof, from plant leaves, stems, fruit, roots or a combination thereof. Plant matter may comprise a plant or portion thereof which has not been subjected to any processing steps. A portion of a plant may comprise plant matter. However, it is also contemplated that the plant material may be subjected to minimal processing steps as 30 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.

[00264] 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.

[00265] 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.

[00266] 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.

[00267] 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.

[00268] 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 tristeza virus (CTV-p23), p24 of Grapevine leafroll-associated virus-2, (GLRaV-2 p24), p10 of Grapevine virus A, (GVA-p10), p14 of Grapevine virus B (GVB-p14), p10 of Heracleum latent virus (HLV-p10), or p16 of Garlic common latent virus (GCLV-p16). Therefore, a suppressor of silencing, for example, but not limited to, HcPro, TEV -p1/Hc-Pro, BYV-p21, TBSV p19, TCV-CP, CMV-2b, PVX-p25, PVM-p11, PVS-p11, BScV-p16, CTV-p23, GLRaV-2 p24, GBV-p14, HLV-p10, GCLV-p16 or GVA-p10, may be co-expressed along with the nucleic acid sequence encoding the protein of interest to further ensure high levels of protein production within a plant.

[00269] 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, type B, 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, H6, H7, H9 or B.

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.

5 [00270] 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.

[00271] Therefore, the present invention further includes a suitable vector comprising the chimeric construct suitable for use with either stable or transient expression systems. The
10 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
15 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
20 sequence operatively linked to a promoter or regulatory region.

[00272] 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
25 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
30 under conditions that both sequences are expressed. Alternatively, a platform plant comprising one of the nucleotide sequences, for example the sequence encoding the protein that modifies the glycosylation profile of the protein of interest, may be transformed, either transiently or in a

stable manner, with an additional sequence encoding the protein of interest. In this case, the sequence encoding the protein that modifies the glycosylation profile of the protein of interest may be expressed within a desired tissue, during a desired stage of development, or its expression may be induced using an inducible promoter, and the additional sequence encoding the protein of interest may be expressed under similar conditions and in the same tissue, to ensure that the nucleotide sequences are co-expressed.

[00273] The constructs of the present invention can be introduced into plant cells using Ti plasmids, Ri plasmids, plant virus vectors, direct DNA transformation, micro-injection, electroporation, infiltration, and the like. For reviews of such techniques see for example Weissbach and Weissbach, *Methods for Plant Molecular Biology*, Academy Press, New York VIII, pp. 421-463 (1988); Geier and Corey, *Plant Molecular Biology*, 2d Ed. (1988); and Miki and Iyer, Fundamentals of Gene Transfer in Plants. In *Plant Metabolism*, 2d Ed. DT. Dennis, DH Turpin, DD Lefebvre, DB Layzell (eds), Addison-Wesley, Langmans Ltd. London, pp. 561-579 (1997). Other methods include direct DNA uptake, the use of liposomes, electroporation, for example using protoplasts, micro-injection, microprojectiles or whiskers, and vacuum infiltration. See, for example, Bilang, et al. (Gene 100: 247-250 (1991), Scheid et al. (Mol. Gen. Genet. 228: 104-112, 1991), Guerche et al. (Plant Science 52: 111-116, 1987), Neuhaus et al. (Theor. Appl Genet. 75: 30-36, 1987), Klein et al., Nature 327: 70-73 (1987); Howell et al. (Science 208: 1265, 1980), Horsch et al. (Science 227: 1229-1231, 1985), DeBlock et al., Plant Physiology 91: 694-701, 1989), Methods for Plant Molecular Biology (Weissbach and Weissbach, eds., Academic Press Inc., 1988), Methods in Plant Molecular Biology (Schuler and Zielinski, eds., Academic Press Inc., 1989), Liu and Lomonosoff (J. Virol Meth, 105:343-348, 2002), U.S. Pat. Nos. 4,945,050; 5,036,006; 5,100,792; 6,403,865; 5,625,136, (all of which are hereby incorporated by reference).

[00274] Transient expression methods may be used to express the constructs of the present invention (see Liu and Lomonosoff, 2002, Journal of Virological Methods, 105:343-348; which is incorporated herein by reference). Alternatively, a vacuum-based transient expression method, as described by Kapila et al. 1997 (incorporated herein by reference) may be used. These methods may include, for example, but are not limited to, a method of Agro-inoculation or Agro-infiltration, however, other transient methods may also be used as noted above. With either Agro-inoculation or Agro-infiltration, a mixture of *Agrobacteria* comprising the desired nucleic acid enter the intercellular spaces of a tissue, for example the leaves, aerial portion of the

plant (including stem, leaves and flower), other portion of the plant (stem, root, flower), or the whole plant. After crossing the epidermis the *Agrobacterium* infect and transfer t-DNA copies into the cells. The t-DNA is episomally transcribed and the mRNA translated, leading to the production of the protein of interest in infected cells, however, the passage of t-DNA inside the nucleus is transient.

[00275] 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. 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.

[00276] The recombinant HA VLPs of the present invention can be used in 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, Chiron, Roche, MedImmune, GlaxoSmithKline, Novartis, Sanofi-Aventis, Serono, Shire Pharmaceuticals and the like.

[00277] If desired, the VLPs of the present invention may be admixed with a suitable adjuvant as would be known to one of skill in the art. Furthermore, the VLP may be used in a vaccine composition comprising an effective dose of the VLP for the treatment of a target organism, as defined above. Furthermore, the VLP produced according to the present invention may be combined with VLPs obtained using different influenza proteins, for example, neuraminidase (NA).

[00278] 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.

[00279] 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).

[00280] As shown in Figures 26A and 26 B a subject administered A/Indonesia/5/05 H5 VLPs is 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 H5 VLPs, but challenged with Turkey H5N1, exhibited significant loss of body mass, and several subject died.

[00281] 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.

[00282] 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, A/Brisbane/50/2007, A/Solomon Islands 3/2006, A/Brisbane/10/2007, A/Wisconsin/67/2005, B/Malaysia/2506/2005, B/Florida/4/2006, A/Singapore/1/57, A/Anhui/1/2005, A/Vietnam/1194/2004, A/Teal/HongKong/W312/97, A/Equine/Prague/56 or A/ HongKong/1073/99. 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.

[00283] 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 (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),

- 5 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)).

- [00284] The choice of combination of strains and subtypes may depend on the geographical area of the subjects likely to be exposed to influenza, proximity of animal species to a human population to be immunized (e.g. species of waterfowl, agricultural animals such as swine, etc) and the strains they carry, are exposed to or are likely to be exposed to, predictions of antigenic drift within subtypes or strains, or combinations of these factors. Examples of combinations used in past years are available (see URL: who.int/csr/disease/influenza/vaccine_recommendations1/en) . Some or all of these strains may be employed in the combinations shown, or in other combinations, in the production of a vaccine composition.

[00285] More particularly, exemplary combinations may include VLPs from two or more strains or subtypes selected from the group comprising: 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.

- 20 [00286] 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/Guizhou/337/2006, A/goose/Guizhou/337/2006 – like virus, A/chicken/Shanxi/2/2006, or A/chicken/Shanxi/2/2006-like virus.

- 25 [00287] Another exemplary combination may include VLPs of A/Chicken/Italy/13474/99 (H7 type) or A/Chicken/British Columbia/04 (H7N3) strains of influenza.

- [00288] 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.

[00289] 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.

[00290] Therefore, the invention provides for compositions comprising VLPs of two or more strains or subtypes.

[00291] 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 presence or absence of adjuvant are similar (Figure 23A).

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

Table 5: Sequence description for sequence identifiers.

SEQ ID No	Sequence Description	In Disclosure
1	N terminal H1 fragment	Figure 4a
2	C terminal H1 fragment	Figure 4b
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

SEQ ID No	Sequence Description	In Disclosure
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
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 5
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	<i>M. Sativa</i> protein disulfide isomerase GenBank Accession No. Z11499	Figure 17
35	A/PuertoRico/8/34 (H1N1) GenBank Accession No. NC_002016.1	Figure 18

SEQ ID No	Sequence Description	In Disclosure
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
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

SEQ ID No	Sequence Description	In Disclosure
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
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

SEQ ID No	Sequence Description	In Disclosure
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
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

SEQ ID No	Sequence Description	In Disclosure
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	Figure 63
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 amino acid sequence 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 66
76	Amino acid sequence of H1 Puerto Rico (NC_0409878.1) encoded by SEQ ID NO: 35	Figure 67
77	pBinPlus.2613c	AGGAAGGGAAGAAA GCGAAAGGAG
78	Mut-ATG115.r	GTGCCGAAGCACGAT CTGACAACGTTGAAG ATCGCTC AC GCAAGA AAGACAAGAGA
79	Mut-ATG161.c	GTTGTCAGATCGTGC TTCGGCACCAGTACA ACG TTTTCTTTCACTG AAGCGA
80	LC-C5-1.110r	TCTCCTGGAGTCACA GACAGGGTGG
81	Expression cassette number 828, from PacI (upstream promoter) to AscI (immediately downstream NOS terminator).	Figure 68

SEQ ID No	Sequence Description	In Disclosure
82	SpPDI-HA(Ind).c	GTTCTTCTCAGATCT TCGCTGATCAGATTT GCATTGGTTACCATG CA
83	Construct number 663, from HindIII (in the multiple cloning site, upstream Plastocyanine promoter) to EcoRI (immediately downstream Plastocynine terminator).	Figure 69
84	SpPDI-H1B.c	<u>TTCTCAGATCTTCG</u> <u>CTGACACAATATGT</u> ATAGGCTACCATGC TAACAAC
85	SacI-H1B.r	CTTAG <u>GAGCTCT</u> TAG ATGCATATTCTACA CTGTAAAGACCCAT TGGAA
86	Construct number 787, from HindIII (in the multiple cloning site, upstream Plastocyanine promoter) to EcoRI (immediately downstream Plastocynine terminator)	Figure 70
87	H3B-SpPDI.r	TGTCATTTCCGGGA AGTTTTTG <u>GAGCGAA</u> <u>GATCTGAGAAGGA</u> <u>ACCA</u>
88	SpPDI-H3B.c	<u>TCTCAGATCTTCG</u> <u>CTCAAAA</u> ACTTCCC GGAAATGACAACA GCACG
89	H3(A-Bri).982r	TTGCTTAACATATC TGGGACAGG
90	Construct number 790, from HindIII (in the multiple cloning site, upstream Plastocyanine promoter) to EcoRI (immediately downstream Plastocynine terminator).	Figure 7
91	HBf-SpPDI.r	GTTATTCCAGTGCA GATTCGATCAGCGA <u>AGATCTGAGAAGG</u> <u>AACCAACAC</u>
92	SpPDI-HBf.c	<u>CAGATCTTCGCTGA</u> <u>TCGAATCTGCACTG</u> GAATAACATCTTCA AACTCACC
93	Plaster80r	CAAATAGTATTTCA TAACAACAACGATT

SEQ ID No	Sequence Description	In Disclosure
94	Construct number 798, from HindIII (in the multiple cloning site, upstream Plastocyanine promoter) to EcoRI (immediately downstream Plastocyanine terminator).	Figure 72
95	Apal-SpPDI.c	TTGTC <u>GGGCCC</u> CAT GGCGAAAAACGTT GCGATTTTCGGCTT ATTGT
96	StuI-H1(A-NC).r	AAAAT <u>AGGCCTT</u> T AGATGCATATTCTA CACTGCAAAGACCC A
97	Construct number 580, from PacI (upstream 35S promoter) to AscI (immediately downstream NOS terminator).	Figure 73
98	Apal-H5 (A-Indo).1c	TGTC <u>GGGCCC</u> CATG GAGAAAATAGTGCT TCTTCTTGCAAT
99	H5 (A-Indo)-StuI.1707r	AAAT <u>AGGCCTT</u> TA AATGCAAATTCTGC ATTGTAACGA
100	Construct number 685, from PacI (upstream 35S promoter) to AscI (immediately downstream NOS terminator).	Figure 74
101	Construct number 686, from PacI (upstream 35S promoter) to AscI (immediately downstream NOS terminator)	Figure 75
102	Apal-H1B.c	TGTC <u>GGGCCC</u> CATG AAAGTAAACTACT GGTCCTGTTATGCA CATT
103	StuI-H2B.r	AAAT <u>AGGCCTT</u> TA GATGCATATTCTAC ACTGTAAAGACCCA TTGGA
104	Construct 732, from PacI (upstream 35S promoter) to AscI (immediately downstream NOS terminator).	Figure 76
105	Construct number 733, from PacI (upstream 35S promoter) to AscI (immediately downstream NOS terminator).	Figure 77
106	Apal-H3B.c	TTGTC <u>GGGCCC</u> CAT GAAGACTATCATTG CTTTGAGCTACATT CTATGTC

SEQ ID No	Sequence Description	In Disclosure
107	StuI-H3B.r	AAAAT <u>AGGCCTTC</u> AAATGCAAATGTTG CACCTAATGTTGCC TTT
108	Construct number 735, from PacI (upstream 35S promoter) to AscI (immediately downstream NOS terminator).	Figure 78
109	Construct number 736, from PacI (upstream 35S promoter) to AscI (immediately downstream NOS terminator).	Figure 79
110	Apl-HBF.c	TTGTC <u>GGGCCCCAT</u> GAAGGCAATAATTG TACTACTCATGGTA GTAAC
111	StuI-HBF.r	AAAAT <u>AGGCCTTT</u> ATAGACAGATGGA GCATGAAACGTTGT CTCTGG
112	Construct number 738, from PacI (upstream 35S promoter) to AscI (immediately downstream NOS terminator).	Figure 80
113	Construct number 739, from PacI (upstream 35S promoter) to AscI (immediately downstream NOS terminator).	Figure 81
114	<i>M. sativa</i> Msj1 coding sequence	Figure 82
115	Hsp-40Luz.1c	ATGTTTGGGCGCGG ACCAAC
116	Hsp40Luz-SacI.1272r	AGCT <u>GAGCTCCTAC</u> TGTTGAGCGCATTG CAC
117	Hsp40Luz- <u>Plasto</u> .r	GTTGGTCCGCGCCC AAACATTTTCTCTC <u>AAGATGAT</u>
118	Hsp70Ara.1c	ATGTCGGGTAAAGG AGAAGGA
119	Hsp70Ara-SacI.1956r	AGCT <u>GAGCTCTTAG</u> TCGACCTCCTCGAT CTTAG
120	Hsp70Ara- <u>Plasto</u> .r	TCCTTCTCCTTTACC CGACATTTTCTCTC <u>AAGATGAT</u>
121	Construct number R850, from HindIII (in the multiple cloning site, upstream promoter) to EcoRI (immediately downstream NOS terminator).	Figure 83

SEQ ID No	Sequence Description	In Disclosure
122	Construct number R860, from HindIII (in the multiple cloning site, upstream promoter) to EcoRI (immediately downstream NOS terminator)	Figure 84
123	Construct number R870, from HindIII (in the multiple cloning site, upstream promoter) to EcoRI (immediately downstream NOS terminator).	Figure 85
124	supP19-plasto.r	CCTTGTATAGCTCG TTCCATTTTCTCTCA AGATG
125	supP19-1c	ATGGAACGAGCTAT ACAAGG
126	SupP19-SacI.r	AGTCGAGCTCTTAC TCGCTTTCTTTTTCG AAG

[00292] The invention will now be described in detail by way of reference only to the following non-limiting examples.

Methods and Materials

1. Assembly of plastocyanin-based expression cassettes for native HA

5 [00293] 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 plastocyanin promoter and 5'UTR sequences were amplified from alfalfa genomic DNA using oligonucleotide primers XmaI-pPlas.c (SEQ ID NO: 10 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 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 15 (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 pCAMBIAPlasto.

[00294] A fragment encoding hemagglutinin from influenza strain A/Indonesia/5/05 (H5N1; Acc. No. LANL ISDN125873) was synthesized by Epoch Biolabs (Sugar Land, TX, 20 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).

[00295] Hemagglutinin expression cassettes number 774 to 785 were assembled as follows. A synthetic fragment was synthesized comprising the complete hemagglutinin coding sequence (from ATG to stop) flanked in 3' by alfalfa plastocyanin gene sequences corresponding to the first 84 nucleotides upstream of the plastocyanin ATG and ending with a DraIII restriction site. The synthetic fragments also comprised a SacI site immediately after the stop codon.

[00296] Synthetic hemagglutinin fragments were synthesized by Top Gene Technologies (Montreal, QC, Canada) and Epoch Biolabs (Sugar Land, TX, USA). The fragment synthesized are presented in figures 28 to 39 and correspond to SEQ ID NO:36 to SEQ ID NO:47. For the assembly of the complete expression cassettes, the synthetic fragments were digested with DraIII and SacI and cloned into pCAMBIAPlasto previously digested with the same enzymes. Table 6 presents the cassettes produced with the corresponding HA and other references in the text.

[00297] Table 6: Hemagglutinin expression cassettes assembled from DraIII-SacI synthetic fragments.

Cassette number	Corresponding HA	Synthetic fragment		Complete cassette	
		Figure	Synthetic fragment SEQ ID NO	Figure	Final cassette SEQ ID NO
774	HA of A/Brisbane/59/2007 (H1N1)	28	36	53	62
775	HA of A/Solomon Islands 3/2006 (H1N1)	29	37	54	63
776	HA of A/Brisbane 10/2007 (H3N2)	30	38	60	69
777	HA of A/Wisconsin/67/2005 (H3N2)	31	39	61	70
778	HA of B/Malaysia/2506/2004	32	40	63	72
779	HA of B/Florida/4/2006	33	41	64	73
780	HA of A/Singapore/1/57 (H2N2)	34	42	55	64
781	HA of A/Anhui/1/2005 (H5N1)	35	43	56	65
782	HA of A/Vietnam/1194/2004 (H5N1)	36	44	57	66
783	HA of A/Teal/HongKong/W312/97 (H6N1)	37	45	58	67
784	HA of A/Equine/Prague/56 (H7N7)	38	46	62	71
785	HA of A/HongKong/1073/99 (H9N2)	39	47	59	68

Assembly of plastocyanin-based PDISP/HA-fusion expression cassettes

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H1 A/New Caledonia/20/99 (construct number 540)

[00298] 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).

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[00299] 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).

H5 A/Indonesia/5/2005 (construct number 663)

[00300] The signal peptide of alfalfa protein disulfide isomerase (PDISP) (nucleotides 32-103; Accession No. Z11499; SEQ ID NO: 34; Figure 17) was linked to the HA0 coding sequence of H5 from A/Indonesia/5/2005 as follows. The H5 coding sequence was amplified with primers SpPDI-HA(Ind).c (SEQ ID NO:82) and HA(Ind)-SacI.r (SEQ ID NO: 7; Figure 7D) using construct number 660 (SEQ ID NO:60; Figure 51) as template. The resulting fragment consisted in the H5 coding sequence flanked, in 5', by the last nucleotides encoding PDISP (including a BglII restriction site) and, in 3', by a SacI restriction site. The fragment was digested with BglII and SacI and cloned into construct number 540 (SEQ ID NO:61; Figure 52) previously digested with the same restriction enzymes. The final cassette, named construct number 663 (SEQ ID NO:83), is presented in Figure 69.

H1 A/Brisbane/59/2007 (Construct 787)

[00301] The signal peptide of alfalfa protein disulfide isomerase (PDISP) (nucleotides 32-103; Accession No. Z11499; SEQ ID NO: 34; Figure 17) was linked to the HA0 coding sequence of H1 from A/Brisbane/59/2007 as follows. The H1 coding sequence was amplified with primers SpPDI-H1B.c (SEQ ID NO: 84) and SacI-H1B.r (SEQ ID NO:85) using construct 774 (SEQ ID NO:62; Figure 53) as template. The resulting fragment consisted in the H1 coding sequence flanked, in 5', by the last nucleotides encoding PDISP (including a BglII restriction site) and, in 3', by a SacI restriction site. The fragment was digested with BglII and SacI and cloned into construct number 540 (SEQ ID NO:61; Figure 52) previously digested with the same

restriction enzymes. The final cassette, named construct number 787 (SEQ ID NO:86), is presented in Figure 70.

H3 A/Brisbane/10/2007 (construct number 790)

[00302] The signal peptide of alfalfa protein disulfide isomerase (PDISP) (nucleotides 32-103; Accession No. Z11499; SEQ ID NO: 34; Figure 17) was linked to the HA0 coding sequence of H3 from A/Brisbane/10/2007 as follows. PDISP was linked to the H3 coding sequence by the PCR-based ligation method presented in Darveau et al. (Methods in Neuroscience 26: 77-85(1995)). In a first round of PCR, a segment of the plastocyanine promoter fused to PDISP was amplified using primers Plasto-443c (SEQ ID NO: 4; Figure 7A) and H3B-SpPDI.r (SEQ ID NO:87) with construct 540 (SEQ ID NO:61; Figure 52) as template. In parallel, another fragment containing a portion of the coding sequence of H3 A/Brisbane/10/2007 (from codon 17 to the SpeI restriction site) was amplified with primers SpPDI-H3B.c (SEQ ID NO:88) and H3(A-Bri).982r (SEQ ID NO:89) using construct 776 (SEQ ID NO:69; Figure 60) as template. Amplification products were then mixed and used as template for a second round of amplification (assembling reaction) with primers Plasto-443c (SEQ ID NO: 4; Figure 7A) and H3(A-Bri).982r (SEQ ID NO:89). The resulting fragment was digested with BamHI (in the plastocyanin promoter) and SpeI (in the H3 coding sequence) and cloned into construct number 776 (SEQ ID NO:69; Figure 60), previously digested with the same restriction enzymes to give construct number 790 (SEQ ID NO:90). The construct is presented in Figure 71.

HA B/Florida/4/2006 (construct number 798)

[00303] The signal peptide of alfalfa protein disulfide isomerase (PDISP) (nucleotides 32-103; Accession No. Z11499; SEQ ID NO: 34; Figure 17) was linked to the HA0 coding sequence of HA from HA B/Florida/4/2006 by the PCR-based ligation method presented in Darveau et al. (Methods in Neuroscience 26: 77-85(1995)). In a first round of amplification, a portion of the plastocyanin promoter fused to the PDISP was amplified using primers Plasto-443c (SEQ ID NO: 4; Figure 7A) and HBF-SpPDI.r (SEQ ID NO:91) with construct number 540 (SEQ ID NO:61; Figure 52) as template. In parallel, another fragment containing a portion of the coding sequence of HB B/Flo fused to the plastocyanin terminator was amplified with primers SpPDI-HBF.c (SEQ ID NO:92) and Plaster80r (SEQ ID NO:93) using construct number 779 (SEQ ID NO:73; Figure 64) as template. PCR products were then mixed and used as template for

a second round of amplification (assembling reaction) with primers Plasto-443c (SEQ ID NO: 4; Figure 7A) and Plaster80r (SEQ ID NO:93). The resulting fragment was digested with BamHI (in the plastocyanin promoter) and AflIII (in the HA B/Florida/4/2006 coding sequence) and cloned into construct number 779 (SEQ ID NO:73; Figure 64), previously digested with the same restriction enzymes to give construct number 798 (SEQ ID NO:94). The resulting expression cassette is presented in Figure 72.

Assembly of CPMV-*HT*-based expression cassettes

[00304] CPMV-*HT* expression cassettes use the 35S promoter to control the expression of an mRNA comprising a coding sequence of interest flanked, in 5' by nucleotides 1–512 from the Cowpea mosaic virus (CPMV) RNA2 with mutated ATG at positions 115 and 161 and in 3', by nucleotides 3330–3481 from the CPMV RNA2 (corresponding to the 3' UTR) followed by the NOS terminator. Plasmid pBD-C5-1LC, (Sainsbury et al. 2008; Plant Biotechnology Journal 6: 82-92 and PCT Publication WO 2007/135480), was used for the assembly of CPMV-*HT*-based hemagglutinin expression cassettes. The mutation of ATGs at position 115 and 161 of the CPMV RNA2 was done using a PCR-based ligation method presented in Darveau et al. (Methods in Neuroscience 26: 77-85 (1995)). Two separate PCRs were performed using pBD-C5-1LC as template. The primers for the first amplification are pBinPlus.2613c (SEQ ID NO: 77) and Mut-ATG115.r (SEQ ID NO: 78). The primers for the second amplification were Mut-ATG161.c (SEQ ID NO: 79) and LC-C5-1.110r (SEQ ID NO: 80). The two obtained fragments are then mixed and used as template for a third amplification using pBinPlus.2613c (SEQ ID NO: 77) and LC-C5-1.110r (SEQ ID NO: 80) as primers. Resulting fragment is digested with PacI and ApaI and cloned into pBD-C5-1LC digested with the same enzyme. The sequence of the expression cassette generated, named 828, is presented in Figure 68 (SEQ ID NO: 81).

Assembly of SpPDI-H1 A/New Caledonia/20/99 in CPMV-*HT* expression cassette (construct number 580).

[00305] A sequence encoding alfalfa PDI signal peptide fused to HA0 from H1 A/New Caledonia/20/99 was cloned into CPMV-*HT* as follows. Restriction sites ApaI (immediately upstream of initial ATG) and StuI (immediately downstream stop codon) were added to the hemagglutinin coding sequence by performing a PCR amplification with primers ApaI-SpPDI.c (SEQ ID NO: 95) and StuI-H1(A-NC).r (SEQ ID NO: 96) using construct number 540 (SEQ ID NO:61; Figure 52) as template. Resulting fragment was digested with ApaI and StuI restriction

enzymes and cloned into construct number 828 (SEQ ID NO: 81) digested with the same enzymes. Resulting cassette was named construct number 580 (SEQ ID NO: 97).

Assembly of H5 A/Indonesia/5/2005 in CPMV-*HT* expression cassette (construct number 685).

[00306] The coding sequence of H5 from A/Indonesia/5/2005 was cloned into CPMV-*HT* as follows. Restriction sites ApaI (immediately upstream ATG) and StuI (immediately downstream stop codon) were added to the hemagglutinin coding sequence by performing a PCR amplification with primers ApaI-H5 (A-Indo).1c (SEQ ID NO: 98) and H5 (A-Indo)-StuI.1707r (SEQ ID NO: 99) using construct number 660 (SEQ ID NO:60; Figure 51) as template.

Resulting fragment was digested with ApaI and StuI restriction enzymes and cloned into construct number 828 (SEQ ID NO: 81) digested with the same enzymes. Resulting cassette was named construct number 685 (SEQ ID NO:100).

Assembly of SpPDI-H5 A/Indonesia/5/2005 in CPMV-*HT* expression cassette (construct number 686).

[00307] A sequence encoding alfalfa PDI signal peptide fused to HA0 from H5 A/Indonesia/5/2005 was cloned into CPMV-*HT* as follows. Restriction sites ApaI (immediately upstream ATG) and StuI (immediately downstream stop codon) were added to the hemagglutinin coding sequence by performing a PCR amplification with primers ApaI-SpPDI.c (SEQ ID NO: 95) and H5 (A-Indo)-StuI.1707r (SEQ ID NO: 99) using construct number 663 (SEQ ID NO: 83) as template. Resulting fragment was digested with ApaI and StuI restriction enzymes and cloned into construct number 828 (SEQ ID NO: 81) digested with the same enzymes. Resulting cassette was named construct number 686 (SEQ ID NO: 101).

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

[00308] The coding sequence of HA from H1 A/Brisbane/59/2007 was cloned into CPMV-*HT* as follows. Restriction sites ApaI (immediately upstream ATG) and StuI (immediately downstream stop codon) were added to the hemagglutinin coding sequence by performing a PCR amplification with primers ApaI-H1B.c (SEQ ID NO: 102) and StuI-H1B.r (SEQ ID NO: 103) using construct number 774 (SEQ ID NO:62; Figure 53) as template. Resulting fragment was digested with ApaI and StuI restriction enzymes and cloned into construct number 828 (SEQ ID NO: 81) digested with the same enzymes. Resulting cassette was named construct number 732 (SEQ ID NO: 104).

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

[00309] A sequence encoding alfalfa PDI signal peptide fused to HA0 from H1 A/Brisbane/59/2007 was cloned into CPMV-*HT* as follows. Restriction sites ApaI (immediately upstream ATG) and StuI (immediately downstream stop codon) were added to the hemagglutinin coding sequence by performing a PCR amplification with primers ApaI-SpPDI.c (SEQ ID NO: 95) and StuI-H1B.r (SEQ ID NO: 103) using construct number 787 (SEQ ID NO: 86) as template. Resulting fragment was digested with ApaI and StuI restriction enzymes and cloned into construct number 828 (SEQ ID NO: 81) digested with the same enzymes. Resulting cassette was named construct number 733 (SEQ ID NO: 105).

Assembly of H3 A/Brisbane/10/2007 in CPMV-*HT* expression cassette (construct number 735).

[00310] The coding sequence of HA from H3 A/Brisbane/10/2007 was cloned into CPMV-*HT* as follows. Restriction sites ApaI (immediately upstream ATG) and StuI (immediately downstream stop codon) were added to the hemagglutinin coding sequence by performing a PCR amplification with primers ApaI-H3B.c (SEQ ID NO:106) and StuI-H3B.r (SEQ ID NO: 107) using construct number 776 (SEQ ID NO:69) as template. Resulting fragment was digested with ApaI and StuI restriction enzymes and cloned into construct number 828 (SEQ ID NO: 81) digested with the same enzymes. Resulting cassette was named construct number 735 (SEQ ID NO: 108).

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

[00311] A sequence encoding alfalfa PDI signal peptide fused to HA0 from H3 A/Brisbane/10/2007 was cloned into CPMV-*HT* as follows. Restriction sites ApaI (immediately upstream ATG) and StuI (immediately downstream stop codon) were added to the hemagglutinin coding sequence by performing a PCR amplification with primers ApaI-SpPDI.c (SEQ ID NO:95) and StuI-H3B.r (SEQ ID NO: 107) using construct number 790 (SEQ ID NO:90) as template. Resulting fragment was digested with ApaI and StuI restriction enzymes and cloned into construct number 828 (SEQ ID NO: 81) digested with the same enzymes. Resulting cassette was named construct number 736 (SEQ ID NO:109).

30 Assembly of HA B/Florida/4/2006 in CPMV-*HT* expression cassette (construct number 738).

[00312] The coding sequence of HA from B/Florida/4/2006 was cloned into CPMV-*HT* as follows. Restriction sites *Apa*I (immediately upstream ATG) and *Stu*I (immediately downstream stop codon) were added to the hemagglutinin coding sequence by performing a PCR amplification with primers **ApaI**-HBF.c (SEQ ID NO: 110) and **StuI**-HBF.r (SEQ ID NO: 111) using construct number 779 (SEQ ID NO:73; Figure 64) as template. Resulting fragment was digested with *Apa*I and *Stu*I restriction enzymes and cloned into construct number 828 (SEQ ID NO: 81) digested with the same enzymes. Resulting cassette was named construct number 738 (SEQ ID NO: 112).

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

[00313] A sequence encoding alfalfa PDI signal peptide fused to HA0 from B/Florida/4/2006 was cloned into CPMV-*HT* as follows. Restriction sites *Apa*I (immediately upstream ATG) and *Stu*I (immediately downstream stop codon) were added to the hemagglutinin coding sequence by performing a PCR amplification with primers **ApaI**-SpPDI.c (SEQ ID NO: 95) and **StuI**-HBF.r (SEQ ID NO: 111) using construct number 798 (SEQ ID NO: 94) as template. Resulting fragment was digested with *Apa*I and *Stu*I restriction enzymes and cloned into construct number 828 (SEQ ID NO: 81) digested with the same enzymes. Resulting cassette was named construct number 739 (SEQ ID NO: 113).

Assembly of chaperone expression cassettes

[00314] Two heat shock protein (Hsp) expression cassettes were assembled. In a first cassette, expression of the *Arabidopsis thaliana* (ecotype Columbia) cytosolic HSP70 (Athsp70-1 in Lin et al. (2001) Cell Stress and Chaperones 6: 201-208) is controlled by a chimeric promoter combining elements of the alfalfa Nitrite reductase (Nir) and alfalfa Plastocyanin promoters (Nir/Plasto). A second cassette comprising the coding region of the alfalfa cytosolic HSP40 (MsJ1; Frugis et al. (1999) Plant Molecular Biology 40: 397-408) under the control of the chimeric Nir/Plasto promoter was also assembled.

[00315] An acceptor plasmid containing the alfalfa Nitrite reductase promoter (Nir), the GUS reporter gene and NOS terminator in plant binary vector was first assembled. Plasmid pNir3K51 (previously described in US Patent No. 6,420,548) was digested with HindIII and EcoRI. The resulting fragment was cloned into pCAMBIA2300 (Cambia, Canberra, Australia) digested by the same restriction enzyme to give pCAMBIA-Nir3K51.

[00316] Coding sequences for Hsp70 and Hsp40 were cloned separately in the acceptor plasmid pCAMBIANir3K51 by the PCR-based ligation method presented in Darveau et al. (Methods in Neuroscience 26:77-85 (1995)).

[00317] For Hsp40, Msj1 coding sequence (SEQ ID NO: 114) was amplified by RT-PCR from alfalfa (ecotype Rangeland) leaf total RNA using primers Hsp40Luz.1c (SEQ ID NO: 115) and Hsp40Luz-SacI.1272r (SEQ ID NO: 116). A second amplification was performed with primers Plasto-443c (SEQ ID NO: 4; Figure 7A) and Hsp40Luz-Plasto.r (SEQ ID NO: 117) with construct 660 (SEQ ID NO: 60; Figure 51) as template. PCR products were then mixed and used as template for a third amplification (assembling reaction) with primers Plasto-443c (SEQ ID NO: 4; Figure 7A) and Hsp40Luz-SacI.1272r (SEQ ID NO: 116). The resulting fragment was digested with HpaI (in the plastocyanin promoter) and cloned into pCAMBIANir3K51, previously digested with HpaI (in the Nir promoter) and SacI, and filled with T4 DNA polymerase to generate blunt ends. Clones obtained were screened for correct orientation and sequenced for sequence integrity. The resulting plasmid, named R850, is presented in Figure 83 (SEQ ID NO: 121). The coding region of the Athsp70-1 was amplified by RT-PCR from Arabidopsis leaf RNA using primers Hsp70Ara.1c (SEQ ID NO: 118) and Hsp70Ara-SacI.1956r (SEQ ID NO: 119). A second amplification was performed with primers Plasto-443c (SEQ ID NO: 4; Figure 7A) and Hsp70Ara-Plasto.r (SEQ ID NO: 120) with construct 660 (SEQ ID NO: 60; Figure 51) as template. PCR products were then mixed and used as template for a third amplification (assembling reaction) with primers Plasto-443c (SEQ ID NO: 4; Figure 7A) and Hsp70ARA-SacI.1956r (SEQ ID NO: 119). The resulting fragment was digested with HpaI (in the plastocyanin promoter) and cloned into pCAMBIANir3K51 digested with HpaI (in the Nir promoter) and SacI and filled with T4 DNA polymerase to generate blunt ends. Clones obtained were screened for correct orientation and sequenced for sequence integrity. The resulting plasmid, named R860, is presented in Figure 84 (SEQ ID NO: 122).

[00318] A dual Hsp expression plasmid was assembled as follows. R860 was digested with BsrBI (downstream the NOS terminator), treated with T4 DNA polymerase to generate a blunt end, and digested with SbfI (upstream the chimeric NIR/Plasto promoter). The resulting fragment (Chimeric Nir/Plasto promoter-HSP70 coding sequence-Nos terminator) was cloned into R850 previously digested with SbfI and SmaI (both located in the multiple cloning site upstream chimeric Nir/Plasto promoter). The resulting plasmid, named R870, is presented in Figure 85 (SEQ ID NO: 123).

Assembly of other expression cassettes

Soluble H1 expression cassette

[00319] 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.

M1 A/Puerto Rico/8/34 expression cassette

[00320] 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 SmaI (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).

HcPro expression cassette

[00321] 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.

P19 expression cassette

[00322] The coding sequence of p19 protein of tomato bushy stunt virus (TBSV) was linked to the alfalfa plastocyanin expression cassette by the PCR-based ligation method presented in Darveau et al. (Methods in Neuroscience 26: 77-85(1995)). In a first round of PCR, a segment of the plastocyanin promoter was amplified using primers Plasto-443c (SEQ ID NO: 4; Figure 7A) and supP19-plasto.r (SEQ ID NO:124) with construct 660 (SEQ ID NO:60; Figure 51) as template. In parallel, another fragment containing the coding sequence of p19 was amplified with primers supP19-1c (SEQ ID NO:125) and SupP19-SacI.r (SEQ ID NO: 126)

using construct 35S:p19 as described in Voinnet et al. (The Plant Journal 33: 949-956 (2003)) as template. Amplification products were then mixed and used as template for a second round of amplification (assembling reaction) with primers Plasto-443c (SEQ ID NO: 4; Figure 7A) and SupP19-SacI.r (SEQ ID NO: 126). The resulting fragment was digested with BamHI (in the plastocyanin promoter) and SacI (at the end of the p19 coding sequence) and cloned into construct number 660 (SEQ ID NO:60; Figure 51), previously digested with the same restriction enzymes to give construct number R472. Plasmid R472 is presented in Figure 86.

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

[00323] *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

[00324] *Agrobacteria* transfected with each construct were grown in a YEB medium supplemented with 10 mM 2-[N-morpholino]ethanesulfonic acid (MES), 20 µM acetosyringone, 50 µg/ml kanamycin and 25 µg/ml of carbenicillin pH5.6 until they reached an OD₆₀₀ between 0.6 and 1.6. *Agrobacterium* suspensions were centrifuged before use and resuspended in infiltration medium (10 mM MgCl₂ and 10 mM MES pH 5.6). Syringe-infiltration was performed as described by Liu and Lomonossoff (2002, *Journal of Virological Methods*, 105:343-348). For vacuum-infiltration, *A. tumefaciens* suspensions were centrifuged, resuspended in the infiltration medium and stored overnight at 4°C. On the day of infiltration, culture batches were diluted in 2.5 culture volumes and allowed to warm before use. Whole plants of *N. benthamiana* or *N. tabacum* were placed upside down in the bacterial suspension in an air-tight stainless steel tank under a vacuum of 20-40 Torr for 2-min. Following syringe or vacuum infiltration, plants were returned to the greenhouse for a 4-5 day incubation period until harvest. Unless otherwise specified, all infiltrations were performed as co-infiltration with AGL1/35S-HcPro in a 1:1 ratio, except for CPMV-HT cassette-bearing strains which were co-infiltrated with strain AGL1/R472 in a 1:1 ratio.

4. Leaf sampling and total protein extraction

[00325] 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 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

[00326] Size exclusion chromatography (SEC) columns of 32 ml Sephacryl™ S-500 high resolution beads (S-500 HR : GE Healthcare, Uppsala, Sweden, Cat. No. 17-0613-10) were packed and equilibrated with equilibration/elution buffer (50 mM Tris pH8, 150 mM NaCl). One and a half millilitre of crude protein extract was loaded onto the column followed by an elution step with 45 mL of equilibration/elution buffer. The elution was collected in fractions of 1.5 mL relative protein content of eluted fractions was monitored by mixing 10 µL of the fraction with 200 µL of diluted Bio-Rad protein dye reagent (Bio-Rad, Hercules, CA). The column was washed with 2 column volumes of 0.2N NaOH followed by 10 column volumes of 50 mM Tris pH8, 150 mM NaCl, 20% ethanol. Each separation was followed by a calibration of the column with Blue Dextran 2000 (GE Healthcare Bio-Science Corp., Piscataway, NJ, USA). Elution profiles of Blue Dextran 2000 and host soluble proteins were compared between each separation to ensure uniformity of the elution profiles between the columns used.

6. Protein Analysis and Immunoblotting

[00327] Protein concentrations were determined by the BCA protein assay (Pierce Biochemicals, Rockport IL). Proteins were separated by SDS-PAGE under reducing conditions and stained with Coomassie Blue. Stained gels were scanned and densitometry analysis performed using ImageJ Software (NIH).

[00328] Proteins from elution fraction from SEC were precipitated with acetone (Bollag et al., 1996), resuspended in 1/5 volume in equilibration/elution buffer and separated by SDS-PAGE under reducing conditions and electrotransferred onto polyvinylene difluoride (PVDF) membranes (Roche Diagnostics Corporation, Indianapolis, IN) for immunodetection. Prior to

immunoblotting, the membranes were blocked with 5% skim milk and 0.1% Tween-20 in Tris-buffered saline (TBS-T) for 16-18h at 4°C.

[00329] 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). Whole, inactivated virus (WIV), used as controls of detection for H1, H3 and B subtypes, were purchased from National Institute for Biological Standards and Control (NIBSC).

Table 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 10-150	4 µg/ml	Goat anti-mouse (JIR 115-035-146)	1:10 000
H1	A/Solomon Islands/3/2006 (H1N1)	Reducing	NIBSC 07/104	1:2000	Rabbit anti-sheep (JIR 313-035-045)	1:10 000
H1	A/New Caledonia/20/99 (H1N1)	Reducing	FII 10-150	4 µg/ml	Goat anti-mouse (JIR 115-035-146)	1:10 000
H2	A/Singapore/1/57 (H2N2)	Non-reducing	NIBSC 00/440	1:1000	Rabbit anti-sheep (JIR 313-035-045)	1:10 000
H3	A/Brisbane/10/2007 (H3N2)	Non-Reducing	TGA AS393	1:4000	Rabbit anti-sheep (JIR 313-035-045)	1:10 000
H3	A/Brisbane/10/2007 (H3N2)	Non-Reducing	NIBSC 08/136	1:1000	Rabbit anti-sheep (JIR 313-035-045)	1:10 000

H3	A/Wisconsin/67/2005 (H3N2)	Non-Reducing	NIBSC 05/236	1:1000	Rabbit anti-sheep (JIR 313-035-045)	1:10 000
H5	A/Indonesia/5/2005 (H5N1)	Reducing	ITC IT-003-005V	1:4000	Goat anti-rabbit (JIR 111-035-144)	1:10 000
H5	A/Anhui/1/2005 (H5N1)	Reducing	NIBSC 07/338	1:750	Rabbit anti-sheep (JIR 313-035-045)	1:10 000
H5	A/Vietnam/1194/2004 (H5N1)	Non-reducing	ITC IT-003-005	1:2000	Goat anti-rabbit (JIR 111-035-144)	1:10 000
H6	A/Teal/Hong Kong/W312/97 (H6N1)	Non-reducing	BEI NR 663	1:500	Rabbit anti-sheep (JIR 313-035-045)	1:10 000
H7	A/Equine/Prague/56 (H7N7)	Non-reducing	NIBSC 02/294	1:1000	Rabbit anti-sheep (JIR 313-035-045)	1:10 000
H9	A/Hong Kong/1073/99 (H9N2)	Reducing	NIBSC 07/146	1:1000	Rabbit anti-sheep (JIR 313-035-045)	1:10 000
B	B/Malaysia/2506/2004	Non-Reducing	NIBSC 07/184	1:2000	Rabbit anti-sheep (JIR 313-035-045)	1:10 000
B	B/Florida/4/2006	Non-Reducing	NIBSC 07/356	1:2000	Rabbit anti-sheep (JIR 313-035-045)	1:10 000

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

NIBSC: National Institute for Biological Standards and Control;

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

BEI NR: Biodefense and emerging infections research resources repository;

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

TGA: Therapeutic Goods Administration, Australia.

[00330] 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
10 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.

5 7. Sucrose gradient ultracentrifugation

[00331] 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
10 to immunological analysis and hemagglutination assays.

8. Electron microscopy

[00332] 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
15 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).

[00333] 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
20 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

25 [00334] 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.

[00335] 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 (A/Indonesia/5/2005) purification

[00336] 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

[00337] 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.

[00338] For intramuscular administration in hind legs, unanaesthetized mice were immunized with either the plant-made H5 VLP (A/Indonesia/5/2005 (H5N1) vaccine (0.1, 1, 5 or 12 µg), or a control hemagglutinin (H5) antigen. The control H5 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.

[00339] 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.

[00340] 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.

[00341] 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

[00342] 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

[00343] 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 .

[00344] Anti-influenza antibody titres may be quantified in ELISA assays using homologous or heterologous inactivated H5N1 viruses.

[00345] 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-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.

[00346] 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 individual samples.

[00347] Data analysis: Statistical analysis (ANOVA) were performed on all data to establish if differences between groups are statistically significant.

Experimental design for lethal challenge(mice)

[00348] One hundred twenty eight mice were randomly divided into sixteen 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.

[00349] For intramuscular administration in hind legs, unanaesthetized mice 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).

[00350] During the immunization period, mice were weighted once a week and observation and monitored for local reactions at the injection site.

[00351] 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.

5 *Blood collection, lung and nasal washes and spleen collection*

[00352] 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 centrifugation at 8000 g for 10 min.

10 [00353] Four weeks after second immunisation, mice were anaesthetized with CO₂ gas and immediately upon termination, cardiac puncture was used to collect blood.

[00354] 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 1 cc 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 μ g of adjuvanted plant-made vaccine or 5 μ g adjuvanted recombinant H5 antigen as well as on mice immunized intranasally with 1 μ g of adjuvanted plant-made vaccine or 1 μ g 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.

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Antibody titers

[00355] 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

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value of the highest dilution that reached an OD value of at least 0.1 higher than that of negative control samples.

[00356] For antibody class determination (IgG1, IgG2a, IgG2b, IgG3, IgM), the titers were evaluated by ELISA as previously described.

5 Hemagglutination inhibition (HI) titers

[00357] 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

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

[00358] 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 (GenBank Accession No. 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.

[00359] *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.

- 5 [00360] 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

- 10 [00361] 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
- 15 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).
- 20 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.

- [00362] 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
- 25 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).

- 30 [00363] 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).

[00364] 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

[00365] 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 ~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

[00366] 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.

[00367] 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.

[00368] 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).

[00369] 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.

[00370] The bioactivity of HA VLPs was confirmed by their capacity to agglutinate turkey red blood cells (data not shown).

[00371] Figure 15D 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.

[00372] 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

[00373] 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). 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

[00374] 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 plasma membranes were similar. Purified VLPs contained the major phospholipids (phosphatidylcholine and phosphatidylethanolamine) and sphingolipids (glucosyl-ceramide) 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 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

[00375] 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 (H5) (Figure 20A).

[00376] 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 H5 with an alum adjuvant (Figure 20B).

[00377] 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 ($<1/10$ for the non-adjuvanted recombinant H5) HI titres.

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

[00378] Figure 21 A, B illustrates the hemagglutination inhibition (HAI) antibody response 14 days following a “boost” with plant-made H5 VLP, or recombinant soluble H5. 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 H5. Increasing doses of H5 VLP provided a modest increase in HAI over the lowest dose.

[00379] 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 H5, 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 H5 antigen (Figure 21A). For the same dose of 5 μ g, VLPs induced HAI titers 20 times higher than the corresponding dose of the control H5 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..

[00380] When administered intramuscularly, all doses of VLPs induced high level of antibodies capable of binding homologous whole inactivated viruses (Figures 20a and 24). No significant difference was found between the plant-made VLP vaccine and the control H5 antigen (except the 12 µg 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 H5 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).

[00381] 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 or HAI), 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.

[00382] 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

[00383] 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.

[00384] 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 H5. 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 [00385] 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 H5 antigen. When administered i.m., 5 µg VLPs induced a mean HAI titer 12 times higher than the corresponding dose of control H5 antigen. Chitosan did not boost the mean HAI level of the control H5 antigen (Figure 22b) while it increased the mean HAI level of mice immunised with 1 µg VLP
10 administered i.n. by a factor of 5-fold.

Example 10: Antibody isotypes

[00386] Mice administered plant-made H5 VLPs or recombinant soluble H5 in the presence or absence of alum as an added adjuvant demonstrate a variety of immunoglobulin isotypes (Figure 23A).

- 15 [00387] In the presence of an added adjuvant, the antibody isotype profiles of VLPs and the recombinant H5 are similar, with IgG1 being the dominant isotype. When VLPs or recombinant H5 are administered without an added adjuvant, IgG1 response is reduced, but remains the dominant isotype response to VLPs, with IgM, IgG2a, IgG2B and IgG3 maintaining similar titers as in the presence of an added adjuvant. IgG1, IgG2a, and IgG2b titers are
20 markedly reduced when recombinant H5 is administered without an added adjuvant (Figure 23A).

[00388] These data, therefore, demonstrate that plant-made VLPs do not require an added adjuvant to elicit a antibody response in a host.

- [00389] Antibody titers against whole inactivated influenza virus strains
25 (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 soluble HA.

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

[00390] 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 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).

[00391] 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 high for VLPs.

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

[00392] Mice that previously had been administered a two-dose regimen of A/Indonesia/5/05 H5 VLPs as described, were subsequently challenged intranasally 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).

15 [00393] 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 animals administered the control antigen (HA) or 1, 5 or 15 ug of Indonesia H5 VLPs survived up to 17 days post-challenge, when the experiment was terminated.

[00394] 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 ug of the Indonesia H5 VLPs before challenge did not lose any appreciable mass during the course of the experiment, and in particular mice administered 5 ug 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 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.

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[00395] 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.

[00396] 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.

[00397] 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 (H1, H2, H3, H5, H6 and H9) structures in plant extracts using size exclusion chromatography

[00398] 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-, AGL1/785- and AGL1/790-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, indicating 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. In Figure 46, H1 from strain A/New Caledonia/20/1999 and H3 from strain A/Brisbane/10/2007 were produced using PDI signal peptide-containing cassettes. The results obtained indicate that replacement of the native signal peptide by that of alfalfa PDI does not affect the ability of HA to assemble into particles.

Example 14: Transient expression of seasonal influenza virus hemagglutinin by agroinfiltration in *N. benthamiana* plants using the wild-type nucleotide sequence

[00399] The ability of the transient expression system to produce seasonal influenza hemagglutinins was determined through the expression of the H1 subtype from strains
 5 A/Brisbane/59/2007 (H1N1) (plasmid #774), A/New Caledonia/20/1999 (H1N1) (plasmid #540) and A/Solomon Islands/3/2006 (H1N1) (plasmid #775), of the H3 subtype from strains A/Brisbane/10/2007 (plasmid #776) and A/Wisconsin/67/2005 (plasmid #777) and of the B type from strains B/Malaysia/2506/2004 (Victoria lineage) (plasmid #778) and B/Florida/4/2006 (Yamagata lineage) (plasmid #779). The hemagglutinin gene coding sequences were first
 10 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), producing *Agrobacterium* strains AGL1/774, AGL1/540, AGL1/775, AGL1/776, AGL1/777, AGL1/778 and AGL1/779, respectively.

15 [00400] *N. benthamiana* plants were infiltrated with AGL1/774, AGL1/540, AGL1/775, AGL1/776, AGL1/777, AGL1/778 and AGL1/779 and the leaves were harvested after a six-day incubation period. To determine whether H1 accumulated in the agroinfiltrated leaves, protein was first extracted from infiltrated leaf tissue and analyzed by Western blotting using anti-HA antibodies (see Table 6 for the antibodies and conditions used for the detection of each HA
 20 subtype). For the HA from H1 strains, a unique band of approximately 72 kDa was detected in extracts (Figure 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. Using these expression and immunodetection strategies, the expression of influenza HA
 25 from H3 subtype or B type was not detected in the crude protein extracts (Figure 47).

Example 15: Transient expression of potential pandemic influenza virus hemagglutinin by agroinfiltration in *N. benthamiana* plants using the wild-type nucleotide sequence

[00401] The ability of the transient expression system to produce potential influenza hemagglutinins was determined through the expression of the H5 subtype from strains
 30 A/Anhui/1/2005 (H5N1) (plasmid #781), A/Indonesia/5/2005 (H5N1) (plasmid #660) and A/Vietnam/1194/2004 (H5N1) (plasmid #782), the H2 subtype from strain A/Singapore/1/1957

(H2N2) (plasmid #780), the H6 from strain A/Teal/Hong Kong/W312/1997 (H6N1) (plasmid #783), the H7 for strain A/Equipe/Prague/1956 (H7N7) (plasmid #784) and finally H9 from strain A/Hong Kong/1073/1999 (H9N2) (plasmid #785). 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), producing *Agrobacterium* strains AGL1/781, AGL1/660, AGL1/782, AGL1/780, AGL1/783, AGL1/784 and AGL1/785.

[00402] *N. benthamiana* plants were infiltrated with AGL1/781, AGL1/660, AGL1/782, AGL1/780, AGL1/784 and AGL1/785, and the leaves were harvested after a six-day incubation period. To determine whether H5 accumulated in the agroinfiltrated leaves, protein was first extracted from infiltrated leaf tissue and analyzed by Western blotting using appropriate anti-HA antibodies (see Table 6 for the antibodies and conditions used for the detection of each HA subtype). A unique band of approximately 72 kDa was detected in extracts of plants transformed with H5 and H2 expression constructs (Figure 48a and b), 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. Using these expression and immunodetection strategies, the expression of influenza HA from H7 and H9 was not detected in the crude protein extracts (Figure 48b).

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

[00403] 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 was then transfected into *Agrobacterium* (AGL1), producing strain AGL1/660.

[00404] *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, proteins were first extracted from infiltrated leaves and analyzed by Western blot 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 HA0 precursor.

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

[00405] 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
10 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 18: Analysis of the immunogenicity results according to CHMP criteria.

15 [00406] 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 -
20 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.

[00407] 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
25 was within normal ranges during the study. The vaccine was safe and tolerated by the animals.

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%
	<i>Mean HI titer</i>		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%
	<i>Mean HI titer</i>		411		465			217		

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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)

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		40%		20%			60%		
	Mean geometric increase		1.9		1.7			2.8		
	% of HI titer of 1/40		40%		20%			40%		
35 (post boost)	% 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% *		

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	30 µg	5 µg ITC
14 (post 1st inj.)	% 4-fold increase in HI titer		40%		20%			80% *		
	Mean geometric increase		1.8		1.3			6.4 *		
	% of HI titer of 1/40		20%		20%			80% *		
35 (post boost)	% 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% *		

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%

Example 19: Selection of hemagglutinin nucleotide sequences

- 5 [00408] The nucleotide sequences of the HA were retrieved from an influenza sequence database (see URL: flu.lanl.gov), or the NCBI influenza virus resource (Bao et al., 2008. J. Virology 82(2): 596-601; 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
- 10 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.). Domains and sub-domains of influenza hemagglutinin are discussed generally in the Description. Domains or subdomains of a first sequence may be
- 15 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	HA1	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
	A/Brisbane/10/2007	ISDN257648	MDCK	N	Y	Y	Y	202: V/G, 210:L/P, 215: del Ala, 242: S/I

	Strain	Sequence database reference No.	Origin	SP	HA1	HA2	DTm	Divergence
	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	partiel	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

B	B/Malaysia/2506/2004	ISDN126672 (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

	Strain	Sequence database reference No.	Origin	SP	HA1	HA2	DTm	Divergence
	B/Malaysia/2506/2004	ISDN231265	Egg	Y	Y	Y	Y	120 K/N 210 T/A
	B/Malaysia/2506/2004	ISDN231557	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	EU124274	Egg	Y	Y	Y	Y	120 K/N 210 T/A
	B/Malaysia/2506/2004	EU124275	MDCK	Y	Y	Y	Y	120 K/N 210 T/A
	B/Malaysia/2506/2004	ISDN124776	MDCK	Y	Y	N	N	120 K/N 210 T/A
B	B/Florida/4/2006	ISDN261649	Egg	Y	Y	Y	N	lacking glycosylation site at position 211; 10 amino acids of DTm/cytoplasmic tail
	B/Florida/4/2006	EU100604	MDCK	N	Y	N	N	
	B/Florida/4/2006	ISDN218061	MDCK	N	Y	N	N	
	B/Florida/4/2006	ISDN285778	Egg	Y	Y	Y	Y	Includes cytoplasmic tail
B	B/Brisbane/3/2007	ISDN256628	Egg	N	Y	N	N	lacking glycosylation site at position 211
	B/Brisbane/3/2007	ISDN263782	Egg	Y	Y	Y	Y	lacking glycosylation site at position 211
	B/Brisbane/3/2007	ISDN263783	MDCK	Y	Y	Y	Y	
H5	A/Viet Nam/1194/2004	ISDN38686 (Vaccine rec.)	?	Y	Y	Y	Y	
	A/Viet Nam/1194/2004	AY651333	?	Y	Y	Y	Y	
	A/Viet Nam/1194/2004	EF541402	?	Y	Y	Y	Y	
H5	A/Anhui1/1/2005	DQ37928 (vaccine rec.)	?	Y	Y	Y	Y	
	A/Anhui1/1/2005	ISDN131465	Egg	Y	Y	Y	Y	
H7	A/Chicken/Ireland/13474/1999	AJ91720	ARN gen	Y	Y	Y	Y	

	Strain	Sequence database reference No.	Origin	SP	HA1	HA2	DTm	Divergence
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	
	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

DTm – complete transmembrane domain Y/N

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

[00409] 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.

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

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

Numbering from the starting M

10

Strain: H1 from A/Brisbane/59/2007

[00410] Position 203 exhibited a variation of aspartic acid (D), isoleucine (I) or asparagine (N).

Strain: H3 from A/Brisbane/10/2007

15 [00411] 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

Strain: H3 from A/Wisconsin/67/2005

- 5 [00412] 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

[00413] Variation at two positions is observed (Table 17). Position 120 is not a glycosylation site; position 210 is involved in glycosylation; this glycosylation is abolished following culture in eggs.

5 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

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

10 [00414] 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. Position 211 is a glycosylation site, and is abolished following culture in eggs.

Strain: H2 from A/Singapore/1/1957

[00415] Sequence variations were observed in 6 positions (Table 18).

15 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

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

[00416] There were no variations observed in the amino acid sequence upon aligning the primary sequences of either of these H5 strains.

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

[00417] Only one entry was available for strain (AF250179).

Strain: H7 from A/Equine/Prague/56

[00418] A total of 2 sequence entries were found in the databases. The entry AB298877 was excluded as it is a laboratory reassortant.

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

[00419] A total of 2 sequence entries were found in the databases. Only one was complete.

Example 20. Transient expression of influenza virus hemagglutinin fused to a signal peptide from a plant secreted protein.

[00420] The effect of signal peptide modification on HA accumulation level for other
 15 hemagglutinins was also investigated through the expression of the A subtype HAs from strains A/Brisbane/59/2007 (H1N1) (plasmid #787), A/New Caledonia/20/1999 (H1N1) (plasmid #540), from strains A/Brisbane/10/2007 (H3N2) (plasmid 790) and A/Indonesia/5/2005 (H5N1) (plasmid #663) and of the B type from strains B/Florida/4/2006 (plasmid #798) fused to the signal peptide (SP; nucleotides 32-103) from of alfalfa protein disulfide isomerase (PDI;
 20 accession No. Z11499; SEQ. ID. NO: 34; Figure 17). The PDI SP-hemagglutinin gene fusions were 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 a pCAMBIA binary plasmid. The plasmids were then transfected into *Agrobacterium* (AGL1), producing *Agrobacterium* strains AGL1/787, AGL1/540,
 25 AGL1/790, AGL1/663 and AGL1/798, respectively.

[00421] *N. benthamiana* plants were infiltrated with AGL1/787, AGL1/540, AGL1/790, AGL1/663 and AGL1/798. In parallel, a series of plants was infiltrated with AGL1/774,

AGL776, AGL1/660 and AGL1/779 for comparison purposes. Leaves were harvested after a six-day incubation period and proteins were extracted from infiltrated leaves and analyzed by Western blot using the appropriate anti-HA antibodies. The expression of HA from H1/Brisbane and H3/Brisbane were considerably improved using the SP from PDI compared to the expression
5 observed for the same HAs with their native signal peptide (Fig. 87b and c, respectively). The expression of a third HA from subtype H1 (strain A/New Caledonia/20/1999) was confirmed using this SP replacement strategy (Fig. 87a). The modification of signal peptide did not lead to substantial increase in HA accumulation for H5 (A/Indonesia/5/2005) (Figure 87d), and no
10 signal was detected for HA from strain B/Florida/4/2006, irrespectively of the signal peptide used for expression (Figure 87e). For all the conditions where the expression of HA was detected, a unique immunoreactive band was observed at a molecular weight of approximately 72 kDa (Fig. 87a to d), corresponding in size to the uncleaved HA0 precursor.

Example 21. HA expression under the control of CPMV-*HT* expression cassette.

15 [00422] An expression cassette CPMV-HT (Sainsbury et al. 2008 Plant Physiology 148: 1212-1218; see also WO 2007/135480) comprising untranslated sequences from the Cowpea mosaic virus (CPMV) RNA2 was used for expression of some hemagglutinins in transgenic plants. HA from A/New Caledonia/20/1999 (H1), A/Brisbane/59/2007 (H1),
20 A/Brisbane/10/2007 (H3), A/Indonesia/5/2005 (H5) and B/Florida/4/2006 (B) were expressed under the control of CPMV-*HT* in *N. benthamiana* plants, agroinfiltrated as described. After incubation, leaves were harvest, extracted and HA contents in protein extracts were compared by Western blot. As shown in Figure 88, the CPMV-HT expression cassette led to higher HA
expression level than the plastocyanin cassette, irrespectively of the signal peptide used. Furthermore, for strain B from B/Florida/4/2006, the use of CPMV-HT expression cassette
25 allowed the detection of HA accumulation which remained undetectable under these immunodetection conditions when expressed under the plastocyanin cassette.

[00423] Table 19: Expression cassette used for expression of influenza hemagglutinins with native or PDI signal peptides.

Agro strain	HA expressed	Signal Peptide	Expression Cassette
AGL1/540	H1 (A/New Caledonia/20/99)	PDI	Plastocyanin
AGL1/580	H1 (A/New Caledonia/20/99)	PDI	CPMV-HT
AGL1/774	H1 (A/Brisbane/59/2007)	native	Plastocyanin
AGL1/787	H1 (A/Brisbane/59/2007)	PDI	Plastocyanin
AGL1/732	H1 (A/Brisbane/59/2007)	native	CPMV-HT
AGL1/776	H3 (A/Brisbane/10/2007)	native	Plastocyanin
AGL1/790	H3 (A/Brisbane/10/2007)	PDI	Plastocyanin
AGL1/735	H3 (A/Brisbane/10/2007)	native	CPMV-HT
AGL1/736	H3 (A/Brisbane/10/2007)	PDI	CPMV-HT
AGL1/660	H5 (A/Indonesia/5/2005)	native	Plastocyanin
AGL1/685	H5 (A/Indonesia/5/2005)	native	CPMV-HT
AGL1/779	B (B/Florida/4/2006)	native	Plastocyanin
AGL1/798	B (B/Florida/4/2006)	PDI	Plastocyanin
AGL1/738	B (B/Florida/4/2006)	native	CPMV-HT
AGL1/739	B (B/Florida/4/2006)	PDI	CPMV-HT

Example 22. Co-expression with Hsp70 and Hsp40 in combination with signal peptide modification.

- 5 [00424] Cytosolic Hsp70 and Hsp40 (construct number R870) of plant origin were co-expressed with H1 New Caledonia (construct number 540) or H3 Brisbane (construct number 790), both bearing a signal peptide of plant origin (alfalfa PDI signal peptide). The co-expression was performed by agroinfiltration of *N. benthamiana* plants with a bacterial suspension containing a mixture (1:1:1 ratio) of AGL1/540, AGL1/R870, AGL1/35SHcPro (For H1) or
- 10 AGL1/790, AGL1/R870 and AGL1/35SHcPro (for H3). Control plants were agroinfiltrated with a mixture (1:2 ratio) of AGL1/540, AGL1/35SHcPro (for H1) or AGL1/790, AGL1/35SHcPro (for H3). After incubation, leaves were harvest, extracted and HA contents in protein extracts were compared by Western blot (Figure 89). In the conditions tested the results obtained indicate that the co-expression of Hsp70 and Hsp40 did not increase hemagglutinin accumulation level
- 15 for H1 New Caledonia. However, for H3 Brisbane, the Western blot clearly indicated that the co-expression of cytosolic Hsp70 and Hsp40 resulted in a significant increase in hemagglutinin accumulation level.

[00425] All citations are hereby incorporated by reference.

[00426] 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.

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WHAT IS CLAIMED IS:

1. A nucleic acid comprising a nucleotide sequence encoding an influenza hemagglutinin (HA) wherein the nucleotide sequence comprises a regulatory element that is operative in a plant, the regulatory element comprising a Cowpea Mosaic Virus (CPMV) regulatory region.
2. A nucleic acid comprising a nucleotide sequence encoding a type B influenza hemagglutinin (HA) operatively linked to a Cowpea Mosaic Virus (CPMV) regulatory region.
3. The nucleic acid of claims 1 or 2, wherein the HA comprises a native or a non-native signal peptide.
4. The nucleic acid of claim 3, wherein the non-native signal peptide is a protein disulfide isomerase signal peptide.
5. The nucleic acid of claim 1, wherein the HA is from a type A influenza selected from the group consisting of H1, H2, H3, H4, H5, H6, H7, H8, H9, H10, H11, H12, H13, H14, H15, and H16.
6. The nucleic acid of claims 1, wherein the HA is from a type A influenza, selected from the group consisting of H1, H2, H3, H5, H6, H7 and H9.
7. The nucleic acid of claim 1, wherein the nucleotide sequence encoding the influenza hemagglutinin (HA) has 70%-100% sequence identity with the nucleotide sequence of SEQ ID NO: 11, SEQ ID NO:12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 23, SEQ ID NO: 24, SEQ ID NO: 25, SEQ ID NO: 26, SEQ ID NO:27, SEQ ID NO: 28, SEQ ID NO: 33, SEQ ID NO: 35, SEQ ID NO: 36, SEQ ID NO:37, SEQ ID NO:38, SEQ ID NO:39, SEQ ID NO:40, SEQ ID NO:41, SEQ ID NO:42, SEQ ID NO:43, SEQ ID NO:44, SEQ ID NO:45, SEQ ID NO:46 or SEQ ID NO:47.
8. A method of producing an influenza virus like particle (VLP) in a transgenic plant comprising:
 - a) introducing the nucleic acid of any one of claims 1 to 7 into a plant, or portion of -a plant by genetic transformation, thereby producing the transgenic plant and

- b) incubating the transgenic plant or portion of the plant under conditions that permit the expression of the nucleic acid, thereby producing the VLP.
9. The method of claim 8, wherein in the step of introducing (step a), the nucleic acid is transiently expressed in the plant.
10. The method of claim 8, wherein, in the step of introducing (step a), the nucleic acid is stably expressed in the plant.
11. The method of any one of claims 7 to 9, wherein, in the step of introducing (step a), a second nucleic acid comprising a nucleotide sequence encoding one or more than one chaperone proteins is introduced to the plant.
12. The method of claim 11, wherein the one or more than one chaperone proteins is selected from the group consisting of Hsp40 and Hsp70.
13. A method of producing an influenza virus like particle (VLP) in a transgenic plant comprising:
- a) providing a transgenic plant, or a portion of a plant, comprising the nucleic acid of any one of claims 1 to 7, and
 - b) incubating the transgenic plant or portion of the plant under conditions that permit the expression of the nucleic acid, thereby producing the VLP
14. The method of claim 8 or 13 further comprising a step of
- c) harvesting the plant and purifying the VLP.
15. The method of claim 14, wherein the VLP range in size from 80-300 nm.
16. A plant cell comprising the nucleic acid of any one of claims 1 to 7.
17. The plant cell of claim 16, further comprising a nucleic acid comprising a nucleotide sequence encoding one or more than one chaperone proteins operatively linked to a regulatory region active in a plant.
18. The plant cell of claim 17, wherein the one or more than one chaperone proteins is selected from the group consisting of Hsp40 and Hsp70.

19. A virus like particle (VLP) produced by the method of any one of claims 14 or 15, comprising an influenza virus hemagglutinin (HA) protein and one or more than one lipid derived from a plant.
20. The VLP of claim 19, wherein the type A influenza, is a subtype selected from the group consisting of H1, H2, H3, H4, H5, H6, H7, H8, H9, H10, H11, H12, H13, H14, H15, and H16.
21. The VLP of claim 20, wherein the HA is from a type A influenza, selected from the group consisting of H1, H2, H3, H5, H6, H7 and H9.
22. A composition comprising an effective dose of the VLP of claim 19 for inducing immunity to an influenza virus in a subject and a pharmaceutically acceptable carrier.
23. A use of the VLP of claim 19 for inducing immunity to an influenza virus infection in a subject.
24. A use of the VLP of claim 19 for the preparation of a medicament for inducing immunity to an influenza virus infection in a subject.
25. The VLP of claim 19 for use in inducing immunity to an influenza virus infection in a subject.
26. The use as defined in any one of claims 23, 24, or the VLP of claim 25, wherein the VLP is administrable to the subject orally, intradermally, intranasally, intramuscularly, intraperitoneally, intravenously, or subcutaneously.
27. A VLP produced by the method of claim 14 or 15, comprising an influenza virus HA bearing plant-specific N-glycans, or modified N-glycans.
28. The VLP of claim 27, wherein the type A influenza is a subtype selected from the group consisting of H1, H2, H3, H4, H5, H6, H7, H8, H9, H10, H11, H12, H13, H14, H15, and H16.
29. The VLP of claim 28, wherein the HA is from a type A influenza, selected from the group consisting of H1, H2, H3, H5, H6, H7 and H9.
30. A composition comprising an effective dose of the VLP of claim 27 for inducing immunity to an influenza virus in a subject, and a pharmaceutically acceptable carrier.

31. A use of the composition of claim 30 for inducing immunity to an influenza virus infection in a subject.
32. A use of the composition of claim 30 for the preparation of a medicament for inducing immunity to an influenza virus infection in a subject.
33. The composition of claim 30 for use in inducing immunity to an influenza virus infection in a subject.
34. The use of the composition as defined in any one of claims 31, 32 or the composition as defined in claim 33, wherein the composition is administrable to a subject orally, intradermally, intranasally, intramuscularly, intraperitoneally, intravenously, or subcutaneously.
35. The VLP of claim 19, wherein the one or more than one lipid is from the group phosphatidycholine, phosphatidylethanolamine, glycosphingolipids, sphingolipid, and a combination thereof.
36. The VLP of any one of claims 19 or 35, further comprising one or more than one phytosterol, sterol, saponin, and a combination thereof.
37. The VLP of claim 36, wherein the phytosterol is from the group stigmasterol, sitosterol, beta-sitosterol, 24-methylcholesterol, cholesterol, and a combination thereof.
38. A nucleic acid comprising a nucleotide sequence having the sequence set forth in SEQ ID NO: 60-73, 81, 83, 86, 90, 94, 97, 100, 101, 104, 105, 108, 109, 112, or 113.
39. The nucleic acid of claim 38, wherein the SEQ ID NO is SEQ ID NO 60 or 61.
40. A method of producing an influenza virus like particle (VLP) in a transgenic plant comprising:
- a) introducing the nucleic acid of claim 38 or 39 into the plant, or portion of the plant, by genetic transformation, thereby producing the transgenic plant and
 - b) incubating the transgenic plant or portion of the plant under conditions that permit the expression of the nucleic acid, thereby producing the VLP.

41. A method of producing an influenza virus like particle (VLP) in a transgenic plant comprising:

a) introducing the nucleic acid of claim 38 and a nucleic acid having the sequence of SEQ ID NO: 122 or SEQ ID NO: 123 into a plant, or portion of the plant, by genetic transformation, thereby producing the transgenic plant and

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

42. The method of any one of claims 8 to 15, 40 or 41 wherein, in the step of introducing (step a), an additional nucleic acid sequence encoding a suppressor of silencing is introduced into the plant.

43. The method of claim 42 wherein, the suppressor of silencing is selected from potato virus HcPro, tobacco etch virus p1/HC-Pro (TEV-p1/HC-Pro), BYV-p21, tomato bushy stunt virus-p19 (TBSV-p19), capsid protein of tomato crinkle virus (TCV-CP), cucumber mosaic virus-2b (CMV-2b), potato virus X-p25 (PVX-p25), potato virus M-p11 (PVM-p11), potato virus S-p11 (PVS-p11), blueberry scorch virus-p16 (BScV-p16), citrus tristeza virus-p23 (CTV-p23), grapevine leafroll-associated virus-2-p14 (GVB-p14), grapevine virus A-p10 (GVA-p10), grapevine virus B-p14 (GVB-p14), heracleum latent virus-p10 (HLV-p10), and garlic common latent virus-p16 (GCLV-p16).

44. The method of any one of claims 8-15, 40-43, wherein, in the step of introducing (step a), a further nucleic acid sequence encoding beta-1.4 galactosyltransferase (GalT), N-acetylglucosaminyltransferase III (GnT-III), GalT- N-acetylglucosaminyl transferase (GalT-GNT1) hybrid enzyme, a GNT1-GnT-III hybrid enzyme, is introduced into the plant.

45. A use of the VLP of any one of claims 19-21, for preparing a sera comprising antibodies specific against influenza hemagglutinin (HA).

46. A polyclonal antibody prepared using the VLP as described in any one of claims 19, 27, 35, 36 or 37, wherein the antibody is specific against influenza hemagglutinin (HA).

47. A plant extract comprising a VLP produced by the method of any one of claims 8 to 15, 40, or 41 to 44.

48. A composition comprising plant extract comprising a VLP produced by the method of any one of claims 8 to 15, 40, or 41 to 44 , and a pharmaceutical acceptable carrier.

49. A plant cell comprising a VLP produced by the method of any one of claims 8 to 15, 40, or 41 to 44.

50. The plant extract of claim 47, the composition of claim 48, or the plant cell of claim 49, for use in inducing immunity to an influenza virus infection in a subject.

51. A use of the plant extract of claim 47, the composition of claim 48, or the plant cell of claim 49 for inducing immunity to an influenza virus infection in a subject, wherein the VLP is suitable for oral administration.

52. A food supplement comprising the plant extract of claim 47 or the plant cell of claim 49.