NEW INFLUENZA VIRUS IMMUNIZING EPITOPE

FIELD OF INVENTION

[0001] 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, most particularly modified influenza antigens that have broad cross-reactivity with other influenza strains.

BACKGROUND OF THE INVENTION

[0002] 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

[0003] Influenza viruses are enveloped viruses that bud from the plasma membrane of infected mammalian cells. They are classified into types A, B, or C, based on the nucleoproteins and matrix protein antigens present. Influenza type A viruses may be further divided into subtypes according to the combination of hemagglutinin (HA) and neuraminidase (NA) surface glycoproteins presented. HA governs the ability of the virus to bind to and penetrate the host cell. NA removes terminal sialic acid residues from glycan chains on host cell and viral surface proteins, which prevents viral aggregation and facilitates virus mobility. Currently, 16 HA (H1-H16) and 9 NA (N1-N9) subtypes are recognized. Each type A influenza virus presents one type of HA and one type of NA glycoprotein. Generally, each subtype exhibits species specificity; for example, all HA and NA subtypes are known to infect birds, while only subtypes H1, H2, H3, H5, H7, H9, H10, N1, N2, N3 and N7 have been shown to infect humans (Horimoto 2006; Suzuki 2005). Influenza viruses comprising H5, H7 and H9 are considered the most highly pathogenic forms of influenza A viruses, and are most likely to cause future pandemics.

[0004] Influenza pandemics are usually caused by highly transmissible 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 1917 and 1920. Presently, the risk of the emergence of a new subtype, or of the transmission to humans of a subtype endemic in animals, is always present. Of particular concern is a highly virulent form of avian influenza (also called "bird flu"), outbreaks of which have been reported in several countries around the world. In many cases, this bird flu can result in mortality rates approaching 100% within 48 hours. The spread of the avian influenza virus (H5N1), first identified in Hong Kong in 1997, to other Asian countries and Europe has been postulated to be linked to the migratory patterns of wild birds.

[0005] The current method of combating influenza in humans is by annual vaccination. The vaccine is usually a combination of several strains that are predicted to be the dominant strains for the coming "flu-season". The prediction is coordinated by the World Health Organization. Generally, the number of vaccine doses produced each year is not sufficient to vaccinate the world's population. For example, Canada and the United-States obtain enough vaccines doses to immunize about one third of their population, while only 17% of the population of the European Union can be vaccinated. It is evident that current worldwide production of influenza vaccine would be insufficient in the face of a worldwide flu pandemic. Even if the necessary annual production could somehow be met in a given year, the dominant strains change from year to year, thus stockpiling at low-need times in the year is not practical. Economical, large scale production of an effective influenza vaccine is of significant interest to government and private industry alike.

[0006] Currently, the most important source viral stocks for use in vaccines are produced in fertilized eggs. The virus particles are harvested, and for an inactivated viral vaccine, disrupted by detergent to inactivate it. Live attenuated vaccines are made of influenza viruses that were adapted for growth at low temperature which means that at normal body temperature, the vaccine is attenuated. Such a vaccine is licensed in USA for use in individuals from 5 to 49 years of age. Inactivated whole virus vaccines are rendered harmless by inactivation with chemical agents and they have been produced in

embryonic eggs or mammalian cell culture. All these types of vaccine show some specific advantages and disadvantages. One advantage of vaccines derived from whole viruses is the type of immunity induced by such vaccines. In general, split vaccines induce a strong antibody response while vaccines made of whole viruses induce both an antibody (humoral) and cellular response. Even though a functional antibody response is a criterion for licensure that correlates with protection induced by a vaccine, there is increasing evidence that a T-cell response is also important in influenza immunity – this may also provide better protection in the elderly.

[0007] 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.

[0008] 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.

[0009] While this method maintains the antigenic epitope and post-translational modifications, there are a number of drawbacks 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 include 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.

[0010] In the case of a pandemic, split vaccine production is slowed 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 since worldwide manufacturing capacity is limited.

[0011] The recent outbreak in Mexico of Influenza type A H1N1 also highlights the urgent medical need to develop rapid methodology for vaccine production of newly emerging strains.

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

[0021] Enveloped viruses may obtain their lipid envelope when 'budding' out of the infected cell and obtain the membrane from the plasma membrane, or from that of an internal organelle. Influenza virus particles and VLPs bud from the plasma membrane of the host cell. In mammalian or baculovirus cell systems, for example, influenza buds from the plasma membrane (Quan et al 2007).

[0022] Only a few enveloped viruses are known to infect plants (for example, members of the Topoviruses and Rhabdoviruses). Of the known plant enveloped viruses, they are characterized by budding from internal membranes of the host cell, and not from the

plasma membrane. Although a small number of recombinant VLPs have been produced in plant hosts, none were derived from the plasma membrane. Current influenza VLP production technologies rely on the co-expression of multiple viral proteins, and this dependence represents a drawback of these technologies since in case of a pandemic and of yearly epidemics, response time is crucial for vaccination. A simpler VLP production system, relying on the expression of only one viral protein is desirable to accelerate the development of vaccine.

[0023] The production of influenza HA VLP in plant based system has been described in WO 2009/009876 that essentially showed that the influenza HA is able to self-assemble in plant host cells and bud from plasma membranes in virus-like particles.

[0024] In order to protect the world population from influenza and to stave off future pandemics, vaccine manufacturers will need to develop effective, rapid methods producing vaccine doses. The current use of fertilized eggs to produce vaccines is insufficient and involves a lengthy process. HA proteins used are specific for each strain and do not cross-react with other strains to provide broader spectrum vaccines thus necessitating constant production or short reaction time once a new strain is identified.

[0025] Certain modifications and/or mutations may be brought to the HA native protein used for producing VLP, such modifications bringing about a hemagglutinin protein that has broader spectrum to induce antibody neutralizing to more than one, or several strains of flu, even after only a single administration.

SUMMARY OF THE INVENTION

[0026] It is an aspect of the invention to provide an improved influenza vaccine.

[0027] It is a further aspect of the invention to provide novel influenza virus-like particles.

[0028] It is a further aspect of the invention to provide an hemagglutinin protein which has been modified to provide a broader spectrum antibody reaction.

[0029] The present invention contemplates a polypeptide having an amino acid residue sequence substantially identical to that of a viral envelope N-linked glycoprotein but that is partially or totally free of N-linked carbohydrates (i.e. has one or more glycosylation site that is abolished when compared to an original native HA sequence), as well as methods of producing and utilizing the polypeptide.

[0030] It is a further aspect of the invention to provide a HA protein wherein one or more of the N-linked glycosylation sites from HA1 domain has been modified/deleted/mutated/removed/abolished to produce influenza VLPs for the preparation of a broad spectrum influenza vaccine.

[0031] Particularly, the HA1 domain comprises amino acids located at positions 1 to 331 as numbered in accordance with strain A/Vietnam/1194/04; **SEQ ID NO.34**).. More particularly, the HA1 domain comprises the globular head portion and the F'2 domain of the protein, corresponding to amino acids between positions 39 to 331 of the protein as numbered in accordance with strain A/Vietnam/1194/04; **SEQ ID NO.34**). Particularly, the glycosylation site that is abolished is originally present on the globular head portion of the protein, particularly corresponding to amino acids located between positions 39 to 273 of SEQ ID No.34. More particularly, the abolished glycosylation site is originally located in the F'2 domain of the protein, particularly corresponding to amino acids located between positions 274-331 of SEQ ID NO.34.

[0032] The present invention provides for amino acid substitutions in the hemagglutinin (NA) molecule of influenza A that can alter the antigenicity and immunogenicity of the HA. These substitutions may alter antigenic sites by altering receptor specificity and/or antibody-antigen binding. In variety of embodiments, the increased antigenicity resulting from the substitution may be useful for the production of vaccines with broader cross-reactivity for influenza. Particularly, the amino acid substitution results in molecules with the immunogenicity characteristics of the amino acid substitution of non-asparagine residue of the HA protein at the location corresponding to the receptor binding-site and particularly corresponding to location 154 and/or 165 and/or 286 (wherein numbering is

in accordance with strain A/Vietnam/1194/04; **SEQ ID NO.34**). In particular embodiments, the amino acid substitution removes/deletes/abolishes a glycosylation site.

[0033] The influenza virus increased antigenicity HA molecule may include one or more non-glycosylated amino acid corresponding to positions 154 and/or 165 and/or 286 in H5 HA, where removal of any one of these glycosylation sites results in an increase reactivity with antisera derived from an animal exposed to an influenza virus with a wildtype HA molecule.

[0034] In order to destroy a glycosylation site, the triad signal N-X-S/T (where N is a Asn, X can be any amino acid except Pro, and S/T can be both Ser or Thr) may be modified by protein engineering. The first approach used can be to replace the Asn by another amino acid. The second approach is to replace the S/T amino acid at position n+2 relative to the asparagine to be glycosylated, by any other amino acid residue. An appropriate amino acid used to replace the asparagine, serine or threonine is alanine, but other amino acid can also be used. For example, Asn can be replaced by Leu, Ile, Val, Thr, Ser or Ala. Also, Ser or Thr can be replaced by Ala, Val, Ile or Leu.

[0035] Particularly, the influenza virus increased antigenicity HA molecule may include a non-asparagine amino acid at positions 154 and/or 165 and/or 286 in H5 HA.

[0036] The influenza virus increased antigenicity HA molecule may include HA protein wherein head portion is devoid of N-linked glycosylation sites i.e. all three glycosylation sites have been abolished.

[0037] The influenza virus increased antigenicity HA molecule may include one or more than one glycosylation site that is removed, selected from the group consisting of: N-154, N-165 and N-286 (wherein the numbering is in accordance with strain A/Vietnam/1194/04).

[0038] The present invention provides a modified hemagglutinin (HA) from different influenza strains.

[0039] The present invention also provides a method of producing influenza virus like particles (VLPs) in a non-sialylating host organism comprising:

a) introducing a nucleic acid encoding an influenza hemagglutinin (HA) antigen as defined above, operatively linked to a regulatory region active in a non-sialylating host organism or a portion thereof, and

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

[0040] 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 host, or stably expressed in the host. Furthermore, the VLPs may be purified using, for example, size exclusion chromatography.

[0041] Additionally the present invention relates to a non-sialylating host organism used for the production of virus like particle (VLP) comprising an influenza virus HA protein. Particularly, suitable host capable of producing a VLP, is for example, a plant or a portion thereof, a plant cell, an insect or a portion thereof, or an insect cell, or a yeast or portion thereof or a yeast cell.

[0042] According to the present invention there is provided a nucleic acid comprising a nucleotide sequence encoding a modified influenza HA as defined above operatively linked to a regulatory region active in a non-sialylating host organism. The antigen may be an influenza hemagglutinin (HA) devoid of one or more the N-linked glycosylation sites from the head portion of the molecule (antigenic sites that are normally present in the native sequence).

[0043] The present invention also provides a virus like particle (VLP) comprising an influenza virus HA protein as defined herein and one or more than one host lipid. 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, if the host is a plant, then the virus like particle (VLP) may comprise an influenza virus HA protein and one or more than one plant lipid.

[0044] The invention further provides VLPs that are produced in a plant thereby containing one or more than one lipid of plant origin (generally referred to as "plant lipids").

[0045] The invention further provides VLPs produced in insect cells comprising lipids from the plasma membrane of insect cells (generally referred to as "insect lipids").

[0046] The invention further provides VLPs produced in yeast comprising lipids from the plasma membrane of yeast cells (generally referred to as "yeast lipids").

[0047] Also included in the present invention is a composition comprising an effective dose of a VLP comprising an influenza virus HA protein, one or more than one lipid derived from a non-sialylating host production cell, in admixture with a pharmaceutically acceptable carrier. The pharmaceutically acceptable carrier may be suitable for oral, intradermal, intranasal, intramuscular, intraperitoneal, intravenous, or subcutaneous administration.

[0048] Further included in the present invention is a vaccine composition comprising an immumologically effective dose of a VLP as defined herein in admixture with a pharmaceutically acceptable carrier with or without the presence of an adjuvant. The vaccine may be administered orally, intradermally, intranasally, intramuscularly, intraperitoneally, intravenously, or subcutaneously. Particularly, the vaccine is administered without the use of an adjuvant.

[0049] The present invention also provides for a method of inducing immunity to an influenza virus infection in a subject, the method comprising administering to the subject the virus like particles comprising an influenza virus HA protein, one or more than one host lipid, and a pharmaceutically acceptable carrier. The virus-like particle may be administered to a subject orally, intradermally, intranasally, intramuscularly, intraperitoneally, intravenously, or subcutaneously.

[0050] The present invention pertains to a method for inducing immunity to influenza virus infection in a subject comprising administering to the subject an effective dose of a vaccine comprising one or more than one VLP as defined herein.

[0051] The subject being treated by the methods as defined above 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. Particularly, the subject may be a human patient or birds in general (including water fowl, migratory birds, poultry such as quail, duck, geese, turkey, chicken), particularly migratory birds or poultry for human consumption (quail, duck, geese, turkey, chicken).

[0052] The present invention also provides for a container such as a syringe as well as kits comprising such a container, all of which comprising the vaccine composition as defined herein.

[0053] This summary of the invention does not necessarily describe all aspects of the invention.

DETAILED DESCRIPTION OF THE INVENTION

BRIEF DESCRIPTION OF THE DRAWINGS

[0054] 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:

Figure 1A represents the localisation of glycosylation sites on the influenza virus HA H5 A/Indonesia/5/05. The amino acids identity, position, and location are indicated by analogy on the structure of the A/Vietnam/1194/04; **SEQ ID NO. 34** (PDB file: 2IBX). The triple mutant has been made by the destruction of the glycosylation sites N154, N165, and N286 located on the globular head. The study from Bright *et al.* (2003) has been used to locate the potential antigenic sites. Glycosylation type has been determined based on what is written in the literature about HAs H1, H3 and H7 (Abe Y. *et al.* (2004); Vigerust DJ *et al.* (2007); and Kuroda *et al.* (1990);

Figure 1B is an illustration of the subdomains of the HA monomer: The F'1 (1-38 as numbered according to A/Vietnam/1194/04; **SEQ ID NO.34**), F'2 (274-331) and F subdomains are represented. The receptor binding site and esterase sub-domains that

together form the globular head (39-273). The fusion peptide is represented as a white box. The TmD and cytotail cannot be seen on any HA structures since only the soluble bromelain products of HAs have been crystallized and structure elucidated;

Figure 2 represents the structures of a monomer of HA from different A subtypes. The lipid bilayer, with its aliphatic counterpart and its polar head is presented as well. Structures taken from Ha et al (Ha Y, Stevens DJ, Skehel JJ, Wiley DC (2002) H5 avian;

Figure 3 shows a sequence of an alfalfa plastocyanin-based expression cassette used for the expression of H1 in accordance with an embodiment of the present invention (SEQ ID NO:8). Protein disulfide isomerase (PDI) signal peptide is underlined. BglII (AGATCT) and SacI (GAGCTC) restriction sites used for cloning are shown in bold;

Figure 4 shows a representation of plasmid 660 assembled for the expression of wild-type HA subtype H5 from A/Indonesia/5/05;

Figure 5 shows a representation of plasmid 680 assembled for the expression of unglycosylated mutated HA subtype H5 from A/Indonesia/5/05;

Figure 6 shows antibody titers against Whole Inactivated Viruses (WIV) after first and second dose. The reactivity of sera from rats immunized with either the wt VLP or the triple mutant VLP (non-glycosylated) was assessed after the first (14 days) or the second immunization (35 days). Immunoreactivity was assessed against several H5N1 viruses;

Figure 7 represents hemagglutination-inhibition (HI) antibody titers after first and second dose. HI titers from rats immunized with the wt or the triple mutant VLP (non-glycosylated) were assessed 14 days after the first (Day 14) or the second (Day 35) immunization. Immunoreactivity was assessed against several H5N1 viruses and one H1N1 virus;

Figure 8 represents the sequence listing for an Influenza HA0;

Figure 9 represents the sequence listing for an Influenza HA protein subtype H2;

Figure 10 represents the sequence listing for an Influenza HA protein subtype H3;

Figure 11 represents the sequence listing for an Influenza HA protein subtype H4;

Figure 12 represents the sequence listing for an Influenza HA protein subtype H5;

Figure 13 represents the sequence listing for an Influenza HA protein subtype H6;

Figure 14 represents the sequence listing for an Influenza HA protein subtype H7;

Figure 15 represents the sequence listing for an Influenza HA protein subtype H8;

Figure 16 represents the sequence listing for an Influenza HA protein subtype H9;

Figure 17 represents the sequence listing for an Influenza HA protein subtype H10;

Figure 18 represents the sequence listing for an Influenza HA protein subtype H11;

Figure 19 represents the sequence listing for an Influenza HA protein subtype H12;

Figure 20 represents the sequence listing for an Influenza HA protein subtype H13;

Figure 21 represents the sequence listing for an Influenza HA protein subtype H14;

Figure 22 represents the sequence listing for an Influenza HA protein subtype H15;

Figure 23 represents the sequence listing for an Influenza HA protein subtype H16;

Figure 24 represents the sequence listing for the 660 pCAMBIA expression vector containing the complete wild type H5 sequence;

Figure 25 A-J represent the sequence listings of primers used for PCR amplification;

Figure 26 represents the sequence listing for 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;

Figure 27 represents the sequence listing for the fragment produced, containing the complete H5 coding region modified to remove all three glycosylation sites, 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;

Figure 28 A-D represent the sequence listings for primers for PCR amplification.

Figure 29 represent amino acid sequence of mature H5 from strain A/Vietnam/1194/04; and

Figure 30A-B represent the nucleic acid and amino acid sequences respectively of mature HA from strain B / Florida/4/2006.

DETAILED DESCRIPTION OF PARTICULAR EMBODIMENTS

[0055] The present invention relates to the production of virus-like particles (VLP). More particularly, the present invention is directed to the production of virus-like particles comprising influenza antigens.

[0056] The following description is of a particular embodiment.

1- HA protein

[0057] As used herein, a "protein" refers generally to a string of amino acids connected by a peptide bond, which may be folded into secondary, tertiary or quaternary structure to achieve a particular morphology. Alternatively, the terms polypeptide, peptide or peptide fragments may be used in a similar context.

[0058] The term "hemagglutinin domain" refers to a peptide comprising either the HA0 precursor polypeptide, or the HA1 and HA2 domains. The hemagglutinin domain does not include the signal peptide, transmembrane domain, or the cytoplasmic tail found in the naturally occurring protein.

[0059] 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.

Structural information on the influenza HAs

[0060] The HA monomer can be subdivided in 2 distinct functional domains, the globular head domain and the stem domain. The correspondence of these domains between the primary sequence and the structure of HA is illustrated at **Figures 1B and 2**. The stem domain is involved in infectivity and pathogenicity of the virus via the extraordinary conformational change it can perform at acidic pH. It is further described as 4 subdomains, the fusion peptide (hydrophobic stretch of 26 amino acids responsible for fusion with the host membrane in the low-pH conformational state); the stem domain itself (that can accommodate 2 extremely different conformations), the transmembrane domain (TmD) (determine the affinity of HA for lipid rafts) the cytoplasmic tail (Ctail) (is involved in secretion of HA). The globular head is divided in 2 subdomains, the receptor binding (RB) domain and the vestigial esterase domain (E). The esterase subdomain is rather buried from the surface of the protein and therefore the majority of antibodies raised against HA binds to the receptor binding domain (represented by the uppermost part of the head in **Figure 2**).

[0061] The term "homotrimer" or "homotrimeric" indicates that an oligomer is formed by three HA protein molecules. HA protein is synthesized as a 75 kDa monomeric precursor protein (HA0), which assembles at the surface into an elongated trimeric protein. For highly pathogenic avian strains, the precursor protein is cleaved intracellularly at a conserved activation cleavage site (also referred to as fusion peptide) into 2 polypeptide chains, HA1 (328 amino acids) and HA2 (221 amino acids; comprising the transmembrane region), linked by a disulfide bond before trimerization occurs. Although this step is central for virus infectivity, it is not essential for the trimerization of the protein. For mammalian and apathogenic avian influenza virus strains, the precursor HA0 is cleaved extracellularly by proteases secreted by cells of the respiratory tract of the host, or by co-infecting bacteria or mycoplasma. 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).

[0062] 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.

[0063] The HA of the present invention may be obtained from any subtype. For example, the HA may be of subtype H1, H2, H3, H4, H5, H6, H7, H8, H9, H10, H11, H12, H13, H14, H15, or H16.

[0064] The present invention includes VLP's comprising HA having modified N-glycans. 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) wherein the native N-linked glycosylation sites have been removed/mutated/deleted/modified to remove the sugar residues that mask the peptidic antigenic sites.

[0065] 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.

[0066] Furthermore, VLPs may be produced that comprise a combination of HA subtypes. For example, VLPs may comprise one or more than one HA from the subtype H1, H2, H3, H4, H5, H6, H7, H8, H9, H10, H11, H12, H13, H14, H15, H16, or a combination thereof. Selection of the combination of HAs may be determined by the intended use of the vaccine prepared from the VLP. For example, a vaccine for use in inoculating birds may comprise any combination of HA subtypes, while VLPs useful for inoculating humans may comprise subtypes from one or more than one of subtypes H1, H2, H3 or H5. However, other HA subtype combinations may be prepared depending

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

[0067] Particularly, 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.

2- Flu subtypes

[0068] The invention includes all types of human influenza virus, including for example, but not limited to the very prevalent A sub-types, and the less common B type, and C type, and to HAs obtained from other influenza subtypes.

[0069] 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:1), H2 (encoded by SEQ ID NO:2), H3 (encoded by SEQ ID NO:3), H4 (encoded by SEQ ID NO:4), H5 (encoded by SEQ ID NO:5), H6 (encoded by SEQ ID NO:6), H7 (encoded by SEQ ID NO:7), H8 (encoded by SEQ ID NO:8), H9 (encoded by SEQ ID NO:9), H10 (encoded by SEQ ID NO:10), H11 (encoded by SEQ ID NO:11), H12 (encoded by SEQ ID NO:12), H13 (encoded by SEQ ID NO:13), H14 (encoded by SEQ ID NO:14), H15 (encoded by SEQ ID NO:15), H16 (encoded by SEQ ID NO:16), 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 humans may comprise any combination of HA subtypes, particularly, one or more than one of subtypes H1, H2, H3, H5, H7, H9, H10, N1, N2, N3 and N7. Particularly, H1, H2, H3, H5.

[0070] However, other HA subtype combinations may be prepared depending upon the use of the inoculum.

3- Method of production

[0071] Furthermore, the present invention provides a method of producing virus like particles (VLPs) in a host. Therefore, the invention provides for VLPs, and a method for producing viral VLPs in a host expression system, from the expression of a single envelope protein. The method involves introducing a nucleic acid encoding an antigen operatively linked to a regulatory region active in the host or a portion thereof, and incubating the host or a portion of the host under conditions that permit the expression of the nucleic acid, thereby producing the VLPs.

[0072] 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 as particularly plant, insect or yeast.

[0073] Particularly, such organism are plants, both monocots and dicots, for example but not limited to corn, cereal plants, wheat, barley, oat, *Nicotiana spp, Brassica spp*, soybean, bean, pea, alfalfa, potato, tomato, ginseng, and *Arabidopsis*.

[0074] Methods for stable transformation, and regeneration of these organisms are established in the art and known to one of skill in the art. The methods of obtaining transformed and regenerated plants are also well known in the art.

[0075] By "transformation" it is meant the stable interspecific transfer of genetic information (nucleotide sequence) that is manifested genotypically, phenotypically or both. The interspecific transfer of genetic information from a chimeric construct to a host may be heritable and the transfer of genetic information considered stable, or the transfer may be transient and the transfer of genetic information is not inheritable.

[0076] Transient expression methods may be used to express the constructs of the present invention (see Liu and Lomonossoff, 2002, Journal of Virological Methods, 105:343-348;

which is incorporated herein by reference). Alternatively, a vacuum-based transient expression method, as described by Kapila *et al.* 1997 (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.

4- Host organism

[0077] The VLPs of the present invention may be produced in a host cell that is characterized by lacking the ability to sialylate proteins, for example lacking sialidase, such as a plant cell, an insect cell, fungi, and other organisms including sponge, coelenterara, annelida, arthoropoda, mollusca, nemathelminthea, trochelmintes, plathelminthes, chaetognatha, tentaculate, chlamydia, spirochetes, gram-positive bacteria, cyanobacteria, archaebacteria, as identified in glycoforum (see, for example, the URL: glycoforum.gr.jp/science/word/evolution/ES-A03E.html).

[0078] 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.

[0079] Particularly, the VLPs of the present invention may be produced in plant cells, a whole plant or portions thereof such as leaf, seeds, or any other plant matter.

[0080] By the term "plant matter", it is meant any material derived from a plant. Plant matter may comprise an entire plant, tissue, cells, or any fraction thereof. Further, plant matter may comprise intracellular plant components, extracellular plant components,

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

[0081] 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.

[0082] 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.

[0083] Also considered part of this invention are transgenic plants, plant cells or seeds containing the chimeric gene construct of the present invention. Methods of regenerating whole plants from plant cells are also known in the art. In general, transformed plant cells are cultured in an appropriate medium, which may contain selective agents such as antibiotics, where selectable markers are used to facilitate 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.

[0084] 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.

[0085] 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.

[0086] Post-transcriptional gene silencing (PTGS) may be involved in limiting expression of transgenes in plants, and co-expression of a suppressor of silencing from the potato virus Y (HcPro) may be used to counteract the specific degradation of

transgene mRNAs (Brigneti *et al.*, 1998). Alternate suppressors of silencing are well known in the art and may be used as described herein (Chiba *et al.*, 2006, Virology 346:7-14; which is incorporated herein by reference), for example but not limited to, TEV - p1/HC-Pro (Tobacco etch virus-p1/HC-Pro), BYV -p21, p19 of Tomato bushy stunt virus (TBSV p19), capsid protein of Tomato crinkle virus (TCV -CP), 2b of Cucumber mosaic virus; CMV-2b), p25 of Potato virus X (PVX-p25), p11 of Potato virus M (PVM-p11), p11 of Potato virus S (PVS-p11), p16 of Blueberry scorch virus, (BScV –p16), p23 of Citrus tristexa virus (CTV-p23), p24 of Grapevine leafroll-associated virus-2, (GLRaV-2 p24), p10 of Grapevine virus A, (GVA-p10), p14 of Grapevine virus B (GVB-p14), p10 of Heracleum latent virus (HLV-p10), or p16 of Garlic common latent virus (GCLV-p16). Therefore, a suppressor of silencing, for example, but not limited to, HcPro, TEV - p1/HC-Pro, BYV-p21, TBSV p19, TCV-CP, CMV-2b, PVX-p25, PVM-p11, PVS-p11, BScV-p16, CTV-p23, GLRaV-2 p24, GBV-p14, HLV-p10, GCLV-p16 or GVA-p10, may be co-expressed along with the nucleic acid sequence encoding the protein of interest to further ensure high levels of protein production within a plant.

[0087] 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 of an adjuvant, and the HAI (hemagglutination inhibition antibody response) determined (**Figure 7**).

[0088] 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 dentritic cells and macrophages and other cells including B and T

lymphocytes in the thymus and liver (Tsuji M,. 2006). Furthermore, in addition to the potential adjuvant effect of the presence of plant lipids, the ability of plant N-glycans to facilitate the capture of glycoprotein antigens by antigen presenting cells (Saint-Jore-Dupas, 2007), may be advantageous of the production of VLPs in plants.

[0089] 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 production/manufacturing systems and that the immune reaction induced by these plant-made VLPs will be stronger when compared to the immune reaction induced by live or attenuated whole virus vaccines.

[0090] Contrary to vaccines made of whole viruses, VLPs provide the advantage as they are non-infectious, thus restrictive biological containment is not as significant an issue as it would be working with a whole, infectious virus, and is not required for production. Plant-made VLPs provide a further advantage again by allowing the expression system to be grown in a greenhouse or field, thus being significantly more economical and suitable for scale-up.

[0091] Additionally, plants do not comprise the enzymes involved in synthesizing and adding sialic acid residues to proteins. VLPs may be produced in the absence of neuraminidase (NA), and there is no need to co-express NA, or to treat the producing cells or extract with sialidase (neuraminidase), to ensure VLP production in plants.

[0092] Particularly, 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 for use as a human vaccine.

5- nucleic acids

[0093] The present invention provides a nucleic acid comprising a nucleotide sequence encoding an influenza virus hemagglutinin (HA) antigen, operatively linked to a regulatory region active in a non-sialylating host organism.

[0094] The present invention describes, but is not limited to, the cloning of a nucleic acid encoding HA, for example but not limited to, a human influenza A virus HA into a host expression vector, and the production of influenza VLPs from the host, suitable for vaccine production. 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.

[0095] The present invention also includes nucleotide sequences H1 (encoded by SEQ ID NO:1), H2 (encoded by SEQ ID NO:2), H3 (encoded by SEQ ID NO:3), H4 (encoded by SEQ ID NO:4), H5 (encoded by SEQ ID NO:5), H6 (encoded by SEQ ID NO:6), H7 (encoded by SEQ ID NO:7), H8 (encoded by SEQ ID NO:8), H9 (encoded by SEQ ID NO:9), H10 (encoded by SEQ ID NO:10), H11 (encoded by SEQ ID NO:11), H12 (encoded by SEQ ID NO:12), H13 (encoded by SEQ ID NO:13), H14 (encoded by SEQ ID NO:14), H15 (encoded by SEQ ID NO:15), and H16 (encoded by SEQ ID NO:16).

[0096] Particularly, the present invention includes nucleotide sequences SEQ ID NO:1; SEQ ID NO:5; SEQ ID NO:7 encoding HA from H1, H5 or H7 respectively; a nucleotide sequence SEQ ID NO:1; SEQ ID NO:5; SEQ ID NO:7, that hybridizes under stringent hybridisation conditions to a nucleic acid that encodes the HA from H1, H5 or H7, respectively; or a nucleotide sequence SEQ ID NO:1; SEQ ID NO:5; SEQ ID NO:7, that hybridizes under stringent hybridization conditions to a complement of a nucleic acid encoding the HA from H1, H5 or H7 respectively; 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 host 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.

[0097] Hybridization under stringent hybridization conditions are 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.

[0098] 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:1), H5 (SEQ ID NO:5) or H7 (SEQ ID NO:7), 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.

[0099] Sequence identity or sequence similarity may be determined using a nucleotide sequence comparison program, such as that provided within DNASIS (for example, using, but not limited to, the following parameters: GAP penalty 5, #of top diagonals 5, fixed GAP penalty 10, k-tuple 2, floating gap 10, and window size 5). However, other methods of alignment of sequences for comparison are well-known in the art for example the algorithms of Smith & Waterman (1981, Adv. Appl. Math. 2:482), Needleman & Wunsch (J. Mol. Biol. 48:443, 1970), Pearson & Lipman (1988, Proc. Nat'l. Acad. Sci.

USA 85:2444), and by computerized implementations of these algorithms (e.g. GAP, BESTFIT, FASTA, and BLAST)., or by manual alignment and visual inspection.

[00100] Therefore, the present invention further includes a suitable vector comprising the chimeric construct suitable for use with either stable or transient expression systems. The genetic information may be also provided within one or more than one construct. For example, a nucleotide sequence encoding a protein of interest may be introduced in one construct, and a second nucleotide sequence encoding a protein that modifies glycosylation of the protein of interest may be introduced using a separate construct. These nucleotide sequences may then be co-expressed within a host. However, a construct comprising a nucleotide sequence encoding both the protein of interest and the protein that modifies glycosylation profile of the protein of interest may also be used. In this case the nucleotide sequence would comprise a first sequence comprising a first nucleic acid sequence encoding the protein of interest operatively linked to a promoter or regulatory region, and a second sequence comprising a second nucleic acid sequence encoding the protein that modifies the glycosylation profile of the protein of interest, the second sequence operatively linked to a promoter or regulatory region.

[00101] By "co-expressed" it is meant that two, or more than two, nucleotide sequences are expressed at about the same time within the host, and within the same tissue of the host. However, the nucleotide sequences need not be expressed at exactly the same time. Rather, the two or more nucleotide sequences are expressed in a manner such that the encoded products have a chance to interact. For example, the protein that modifies glycosylation of the protein of interest may be expressed either before or during the period when the protein of interest is expressed so that modification of the glycosylation of the protein of interest takes place. The two or more than two nucleotide sequences can be co-expressed using a transient expression system, where the two or more sequences are introduced within the host at about the same time under conditions that both sequences are expressed. Alternatively, a platform host 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.

[00102] The constructs of the present invention can be introduced into plant cells using Ti plasmids, Ri plasmids, plant virus vectors, direct DNA transformation, micro-injection, electroporation, etc. For reviews of such techniques see for example Weissbach and Weissbach, Methods for Plant Molecular Biology, Academy Press, New York VIII, pp. 421-463 (1988); Geierson and Corey, Plant Molecular Biology, 2d Ed. (1988); and Miki and Iyer, Fundamentals of Gene Transfer in Plants. In Plant Metabolism, 2d Ed. DT. Dennis, DH Turpin, DD Lefebvre, DB Layzell (eds), Addison Wesly, Langmans Ltd. London, pp. 561-579 (1997). Other methods include direct DNA uptake, the use of liposomes, electroporation, for example using protoplasts, micro-injection, microprojectiles or whiskers, and vacuum infiltration. See, for example, Bilang, *et al.* (Gene 100: 247-250 (1991), Scheid *et al.* (Mol. Gen. Genet. 228: 104-112, 1991), Guerche *et al.* (Plant Science 52: 111-116, 1987), Neuhause *et al.* (Theor. Appl Genet. 75: 30-36, 1987), Klein *et al.*, Nature 327: 70-73 (1987); Howell *et al.* (Science 208: 1265, 1980), Horsch *et al.* (Science 227: 1229-1231, 1985), DeBlock *et al.*, Plant Physiology 91: 694-701, 1989), Methods for Plant Molecular Biology (Weissbach and Weissbach, eds., Academic Press Inc., 1988), Methods in Plant Molecular Biology (Schuler and Zielinski, eds., Academic Press Inc., 1989), Liu and Lomonossoff (J. Virol Meth, 105:343-348, 2002,), U.S. Pat. Nos. 4,945,050; 5,036,006; and 5,100,792, U.S. patent application Ser. Nos. 08/438,666, filed May 10, 1995, and 07/951,715, filed Sep. 25, 1992, (all of which are hereby incorporated by reference).

[00103] By "regulatory region" "regulatory element" or "promoter" it is meant a portion of nucleic acid typically, but not always, upstream of the protein coding region of a gene, which may be comprised of either DNA or RNA, or both DNA and RNA. When a regulatory region is active, and in operative association, or operatively linked, with a gene of interest, this may result in expression of the gene of interest. A regulatory element

may be capable of mediating organ specificity, or controlling developmental or temporal gene activation. A "regulatory region" includes promoter elements, core promoter elements exhibiting a basal promoter activity, elements that are inducible in response to an external stimulus, elements that mediate promoter activity such as negative regulatory elements or transcriptional enhancers. "Regulatory region", as used herein, also includes elements that are active following transcription, for example, regulatory elements that modulate gene expression such as translational and transcriptional enhancers, translational and transcriptional repressors, upstream activating sequences, and mRNA instability determinants. Several of these latter elements may be located proximal to the coding region.

[00104] 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.

[00105] 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 3; US 7,125,978, which is incorporated herein by reference).

[00106] An inducible regulatory region is one that is capable of directly or indirectly activating transcription of one or more DNA sequences or genes in response to an inducer. In the absence of an inducer the DNA sequences or genes will not be transcribed. Typically the protein factor that binds specifically to an inducible regulatory region to activate transcription may be present in an inactive form, which is then directly or indirectly converted to the active form by the inducer. However, the protein factor may also be absent. The inducer can be a chemical agent such as a protein, metabolite, growth regulator, herbicide or phenolic compound or a physiological stress imposed directly by heat, cold, salt, or toxic elements or indirectly through the action of a pathogen or disease agent such as a virus. A plant cell containing an inducible regulatory region may be exposed to an inducer by externally applying the inducer to the cell or plant such as by spraying, watering, heating or similar methods. Inducible regulatory elements may be derived from either plant or non-plant genes (e.g. Gatz, C. and Lenk, I.R.P., 1998, Trends Plant Sci. 3, 352-358; which is incorporated by reference). Examples, of potential inducible promoters include, but not limited to, tetracycline-inducible promoter (Gatz, C.,1997, Ann. Rev. Plant Physiol. Plant Mol. Biol. 48, 89-108; which is incorporated by reference), steroid inducible promoter (Aoyama, T. and Chua, N.H.,1997, Plant J. 2, 397-404; which is incorporated by reference) and ethanol-inducible promoter (Salter, M.G., et al, 1998, Plant Journal 16, 127-132; Caddick, M.X., et al,1998, Nature Biotech. 16, 177-180, which are incorporated by reference) cytokinin inducible IB6 and CKI1 genes (Brandstatter, I. and Kieber, J.J.,1998, Plant Cell 10, 1009-1019; Kakimoto, T., 1996, Science 274, 982-985; which are incorporated by reference) and the auxin inducible

element, DR5 (Ulmasov, T., *et al.*, 1997, Plant Cell 9, 1963-1971; which is incorporated by reference).

[00107] A constitutive regulatory region directs the expression of a gene throughout the various parts of a plant and continuously throughout plant development. Examples of known constitutive regulatory elements include promoters associated with the CaMV 35S transcript. (Odell *et al.*, 1985, Nature, 313: 810-812), the rice actin 1 (Zhang et al, 1991, Plant Cell, 3: 1155-1165), actin 2 (An *et al.*, 1996, Plant J., 10: 107-121), or tms 2 (U.S. 5,428,147, which is incorporated herein by reference), and triosephosphate isomerase 1 (Xu et. al., 1994, Plant Physiol. 106: 459-467) genes, the maize ubiquitin 1 gene (Cornejo et al, 1993, Plant Mol. Biol. 29: 637-646), the Arabidopsis ubiquitin 1 and 6 genes (Holtorf et al, 1995, Plant Mol. Biol. 29: 637-646), and the tobacco translational initiation factor 4A gene (Mandel et al, 1995 Plant Mol. Biol. 29: 995-1004). The term "constitutive" as used herein does not necessarily indicate that a gene under control of the constitutive regulatory region is expressed at the same level in all cell types, but that the gene is expressed in a wide range of cell types even though variation in abundance is often observed.

[00108] 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.

[00109] 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.

[00110] 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.

[00111] 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 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)

[00112] As described herein, promoters comprising enhancer sequences with demonstrated efficiency in leaf expression, have been found to be effective in transient expression. Without wishing to be bound by theory, attachment of upstream regulatory elements of a photosynthetic gene by attachment to the nuclear matrix may mediate strong expression. For example up to -784 from the translation start site of the pea plastocyanin gene may be used mediate strong reporter gene expression.

[00113] The use of a regulatory region from a photosynthetic gene, for example but not limited to a plastocyanin regulatory region (US 7,125,978; which is incorporated herein by reference), or a regulatory region obtained from 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 a; 1986; which is incorporated herein by reference), ST-LS1 (associated with the oxygen-evolving complex of photosystem II, Stockhaus *et al.*1989; which is incorporated herein by reference) may be used in accordance with the present invention.

[00114] To aid in identification of transformed plant cells, the constructs of this invention may be further manipulated to include plant selectable markers. Useful selectable markers include enzymes that provide for resistance to chemicals such as an antibiotic for example, gentamycin, hygromycin, kanamycin, or herbicides such as phosphinothrycin, glyphosate, chlorosulfuron, and the like. Similarly, enzymes providing for production of a compound identifiable by colour change such as GUS (beta-glucuronidase), or luminescence, such as luciferase or GFP, may be used.

[00115] 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 nay be used.

[00116] 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 obtained from 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, Stockhaus *et al.*1989; which is incorporated herein by reference). 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 polyhedron promoter, the gp64 promoter and the like.

[00117] 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, yeast 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.

[00118] The nucleic acid encoding the HA, for example but not limited to, a human influenza A/New Caledonia/20/99 (H1N1) virus HA, or the human influenza A/Indonesia/5/05 virus HA gene 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.

[00119] 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 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 genes or human influenza A/Indonesia/5/05 (H5N1) virus HA0 genes can be used. Additionally, the nucleic acid encoding HA may be chemically synthesized using methods as would known to one of skill in the art.

6- Proteins

[00120] The present invention also includes one or more than one HA protein encoded by nucleotide sequences SEQ ID NO:1; SEQ ID NO:5; SEQ ID NO:7 (encoding HA from H1, H5 or H7, respectively), a nucleotide sequence SEQ ID NO:1; SEQ ID NO:5; SEQ ID NO:7, that hybridizes under stringent hybridisation conditions to a nucleic acid that encodes the HA from H1, H5 or H7, respectively, or a nucleotide sequence SEQ ID

NO:1; SEQ ID NO:5; SEQ ID NO:7, that hybridizes under stringent hybridisation conditions to a compliment of a nucleic acid encoding the HA from H1, H5 or H7, respectively, wherein the nucleotide sequence encodes a hemagglutinin protein that when expressed forms a VLP, and that the VLP induces the production of an antibody.

[00121] Similarly, the present invention includes HAs associated with the following subtypes H1 (encoded by SEQ ID NO:1), H2 (encoded by SEQ ID NO:2), H3 (encoded by SEQ ID NO:3), H4 (encoded by SEQ ID NO:4), H5 (encoded by SEQ ID NO:5), H6 (encoded by SEQ ID NO:6), H7 (encoded by SEQ ID NO:7), H8 (encoded by SEQ ID NO:8), H9 (encoded by SEQ ID NO:9), H10 (encoded by SEQ ID NO:10), H11 (encoded by SEQ ID NO:11), H12 (encoded by SEQ ID NO:12), H13 (encoded by SEQ ID NO:13), H14 (encoded by SEQ ID NO:14), H15 (encoded by SEQ ID NO:15), H16 (encoded by SEQ ID NO:16); and nucleotide sequences that are characterized as having from about 60 to 100% or any amount therebetween sequence identity, particularly from about 70 to 100% of homology 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:1), H2 (SEQ ID NO:2), H3 (SEQ ID NO:3), H4 (SEQ ID NO:4), H5 (SEQ ID NO:5), H6 (SEQ ID NO:6), H7 (SEQ ID NO:7), H8 (SEQ ID NO:8), H9 (SEQ ID NO:9), H10 (SEQ ID NO:10), H11 (SEQ ID NO:11), H12 (SEQ ID NO:12), H13 (SEQ ID NO:13), H14 (SEQ ID NO:14), H15 (SEQ ID NO:15), H16 (SEQ ID NO:16), 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.

7- VLP

[00122] Therefore, the present invention is directed to a VLP comprising one or more than one HA type or subtype.

[00123] The term "virus like particle" (VLP), or "virus-like particles" or "VLPs" refers to structures that self-assemble and comprise structural proteins such as influenza HA protein. VLPs are generally morphologically and antigenically similar to virions produced in an infection, but lack genetic information sufficient to replicate and thus are non-infectious. In some examples, VLPs may comprise a single protein species, or more than one protein species. For VLPs comprising more than one protein species, the protein species may be from the same species of virus, or may comprise a protein from a different species, genus, subfamily or family of virus (as designated by the ICTV nomenclature). In other examples, one or more of the protein species comprising a VLP may be modified from the naturally occurring sequence. VLPs may be produced in suitable host cells including plant and insect host cells. Following extraction from the host cell and upon isolation and further purification under suitable conditions, VLPs may be purified as intact structures.

[00124] 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.

[00125] Generally, the term "lipid" refers to a fat-soluble (lipophilic), naturally-occurring molecules. The term is also used more specifically to refer to fatty-acids and their derivatives (including tri-, di-, and monoglycerides and phospholipids), as well as other fat-soluble sterol-containing metabolites or sterols. Phospholipids are a major component of all biological membranes, along with glycolipids, sterols and proteins. Examples of phospholipids include phosphatidylethanolamine, phosphatidylcholine, phosphatidylinositol, phosphatidylserine, and the like. Examples of sterols include zoosterols (e.g., cholesterol) and phytosterols. Over 200 phytosterols have been identified in various plant species, the most common being campesterol, stigmasterol, ergosterol, brassicasterol, delta-7-stigmasterol, delta-7-avenasterol, daunosterol, sitosterol, 24-methylcholesterol, cholesterol or beta-sitosterol. As one of skill in the art would understand, the lipid composition of the plasma membrane of a cell may vary with the culture or growth conditions of the cell or organism from which the cell is obtained.

[00126] 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.

[00127] The VLPs produced from influenza derived proteins, in accordance with the present invention do not comprise M1 protein. The M1 protein is known to bind RNA (Wakefield and Brownlee, 1989) which is a contaminant of the VLP preparation. The presence of RNA is undesired when obtaining regulatory approval for the VLP product, therefore a VLP preparation lacking RNA may be advantageous.

[00128] 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 or combinations thereof. 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, and one or more than one plant derived lipid, for example 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'.

[00129] In plants, influenza VLPs bud from the plasma membrane, therefore the lipid composition of the VLPs reflects their origin. The VLPs produced according to the present invention comprise HA, 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).

[00130] 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.

[00131] 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.

[00132] 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.

[00133] 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.

8- Composition

[00134] 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. 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.

9- Method of treatment

[00135] The present invention provides for a method of inducing immunity or "provoking an immune response" to an influenza virus infection in a subject, the method comprising administering the composition as defined herein.

[00136] 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.

[00137] 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.

[00138] 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.

[00139] 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).

[00140] 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.

[00141] As shown in **Figures 6** and **7** *in vitro* assays showing cross-reactivity of antibodies raised against the mutated A/Indonesia/5/05 H5 VLPs and other influenza strains such as A/Vietnam/1203/04; A/Anhui/1/05 and A/Turkey/582/06 (all H5N1 strains), whereas it showed less hemagglutination reactivity against the only H1N1 tested (**Figure 7**).

[00142] Significantly, the antibodies produced after a single dose of mutated H5N1 (unglycosylated H5 protein) induced a greater response against all H5 strains tested after 14 days than antibodies produced against the wild-type H5, indicating that this unglycosylated immunogen may provide more rapid response that the wild-type one.

[00143] These data, therefore, demonstrate that plant-made influenza VLPs comprising the mutated H5 hemagglutinin viral protein devoid of N-linked carbohydrates induce an immune response specific for pathogenic influenza strains, and that this response is cross-reactive and may be rapid after one single dose.

10- Subject

[00144] 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, turkey, 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.

[00145] 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, turkey, chicken, camel, canine, dogs, feline, cats, tiger, leopard, civet, mink, stone marten, ferrets, house pets, livestock, mice, rats, seal, whale and the like.

[00146] Particularly, the subject being treated by the method as defined above may be selected from the group comprising humans, primates, horses, pigs, birds (avian) water fowl, migratory birds, quail, duck, geese, chicken, dogs, cats, ferrets, livestock and the like. Particularly, the subject may be a human patient or birds in general (including water fowl, migratory birds, poultry such as quail, duck, geese, turkey, chicken), particularly migratory birds or poultry for human consumption (quail, duck, geese, turkey, chicken). More particularly, the subject is human.

11- Containers, syringes, and kits etc..

[00147] The present invention also provides for a container comprising the composition as defined herein. Particularly, the container contains single unit dose or in multiple dosage form with a preservative agent. More particularly, the container is a syringe "ready-for-use" pre-filled with the composition or the vaccine as defined herein.

[00148] More particularly, the invention also provides for a kit comprising a container comprising the vaccine or composition as defined herein, and instructions on how to use/administer said composition/vaccine.

[00149] The invention will now be described in detail by way of reference only to the following non-limiting examples.

Example 1

Material and methods

1. Mutation of wild-type H5 from A/Indonesia/5/05 (**SEQ ID NO.17**) to obtain mutated unglycosylated H5.

[00150] The triple mutant has been made by removing the glycosylation sites N154, N165, and N286 located on the globular head of wild type HAs, more specifically by replacing the Thr or Ser enclosed in the glycosylation sequence pattern N-X-T/S by an Ala residue. Therefore, the triple mutant contained the following three amino acid replacements: T156A, T167A and S288A (numbered according to the starting SEQ ID NO.17). The three amino acid replacement were performed by PCR-based ligation method presented in Darveau *et al.* (1995) using the wild type HA expression vector (660 construct, Figure 4) as the template.

[00151] Briefly, three PCR amplification were performed in parallel on 660 pCAMBIA expression vector as the template with 3 different pairs of primers:

1) Plato-443c (SEQ ID NO: 18) and HA5-T156A.r (SEQ ID NO:19);

2) HA5-T167A.c (SEQ ID NO:20) and HA5-S288A.r (SEQ ID NO: 21); and

3) HA5-S288A.c (SEQ ID NO:22) and HA(Ind)-SacI.r (SEQ ID NO: 23).

[00152] The amplification products obtained from the three reactions were mixed together and the mixture served as template for a fourth reaction (assembling reaction) using Plato-443c (SEQ ID NO: 18) and HA(Ind)-Sac.r (SEQ ID NO: 23) as primers. The resulting fragment was digested with BamHI (located 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 680, is presented in Figure **5** (**SEQ ID NO.29**).

2. Assembly of expression cassettes

[00153] All manipulations were carried out 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: 24) and SacI-ATG-pPlas.r (SEQ ID NO: 25). 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 (SEQ ID NO: 26) and EcoRI-PlasTer.r (SEQ ID NO: 27), and the product was digested with SacI and EcoRI before being inserted into the same sites of pCAMBIApromoPlasto to create pCAMBIAPlasto.

3. Assembly of H5 expression cassette

[00154] A fragment encoding hemagglutinin from influenza strain A/Indonesia/5/05 (H5N1; Acc. No. LANL ISDN125873) was synthesized by Epoch Biolabs (Sugar Land, TX, USA). The fragment produced, containing the complete H5 coding region (**SEQ ID NO.17**) 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: 28 (and SEQ ID NO.29 in the case of the mutant H5). 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: 30) and SpHA(Ind)-Plasto.r (SEQ ID NO:31) and pCAMBIA promoPlasto as template. In parallel, a second amplification was performed with primers Plasto-SpHA(Ind).c (SEQ ID NO: 6) and HA(Ind)-Sac.r (SEQ ID NO:32) 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) and HA(Ind)-Sac.r (SEQ ID NO: 33) 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 5** whereas the plasmid resulting from the "mutated" H5 protein was named 680.

[00155] 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.

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

[00156] Nicotiana benthamiana plants were grown from seeds in flats filled with a commercial peat moss substrate. The plants were allowed to grow in the greenhouse under a 16/8 photoperiod and a temperature regime of 25°C day/20°C night. Three weeks after seeding, individual plantlets were picked out, transplanted in pots and left to grow in the greenhouse for three additional weeks under the same environmental conditions. Prior to transformation, apical and axillary buds were removed at various times as indicated below, either by pinching the buds from the plant, or by chemically treating the plant

[00157] Agrobacteria transfected with plasmids 660 or 680 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 Nicotiana benthamiana were

placed upside down in the bacterial suspension in an air-tight stainless steel tank under a vacuum of 20-40 Torr for 2-min. Following syringe or vacuum infiltration, plants were returned to the greenhouse for a 4-5 day incubation period until harvest.

5. Leaf sampling and total protein extraction

[00158] 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.

6. Protein Analysis and Immunoblotting

[00159] 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).

[00160] 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.

[00161] Immunoblotting was performed by incubation with the following antibodies: for the detection of H1, a mouse anti-influenza A monoclonal antibody (Fitzgerald Industries International, Concord, MA, USA, Cat. No. 10-I50) (2 µg/ml in 2% skim milk in TBS-Tween 20 0.1%), and for the detection of H5, a rabbit anti-H5 (Vietnam) antibody (Immune Technology, Woodside, NY, USA, Cat No. IT-003-005V) diluted 1/4000 in 2%

skim milk in TBS-Tween 20 0.1%. A peroxidase-conjugated goat anti-mouse IgG (H+L) antibody (Jackson Immunoresearch Laboratories, West Grove, PA, USA, Cat. No. 115-035-146) (diluted 1/12 000 in 2% skim milk in TBS-Tween 20 0.1%) was used as secondary antibody. 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).

[00162] Hemagglutination assay for H5 was based on a method described by Nayak. and Reichl (2004). Briefly, serial double dilutions of the test samples (100 µL) were made in V-bottomed 96-well microtiter plates containing 100 µL PBS, leaving 100 µL of diluted sample per well. One hundred microliters of a 0.25% horse 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.

7. H5 VLP purification

[00163] Frozen 660- or 680-infiltrated leaves of N. benthamiana were homogenized in 1.5 volumes of 50 mM Tris pH 8, NaCl 50 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 4oC and resuspended in PBS pH 7.4 with 0.01% Tween-80 and 0.01% thimerosal. Suspended VLPs were filter-sterilized before use.

8. Animal studies

[00164] Studies on the immune response to influenza VLP administration were performed with 6-8 week old female Wistar rats (Charles River Laboratories). Thirteen rats were randomly divided into three groups ranging from three for the control group to five animals for both the plant-made VLP H5 wild type vaccine (660) and the mutant (680) vaccine groups. 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 14 days following the first immunization.

[00165] For intramuscular administration in hind legs, unanaesthetized rats were immunized with either the plant-made VLP H5 vaccine (15 μg), the plant-made VLP H5 mutant form of the vaccine or PBS.

[00166] All antigen preparations were mixed with Alhydrogel to a final concentration of 1% (alum; Accurate Chemical and Scientific Corporation, Wesbury, NY, US) in a 1:1 volume ratio prior to immunizations.

Blood collection and spleen collection

[00167] Jugular vein blood collection was performed fourteen days after the first immunization and fourteen days after second immunization on anaesthetized animal. Serum was collected by centrifuging at 8000 g for 10 min.

[00168] Three weeks after second immunisation, rats were anaesthetized with CO2 gas and immediately upon termination, cardiac puncture was used to collect blood.

[00169] Spleen collection was performed on rats Collected spleens were placed in RPMI supplemented with gentamycin and mashed in a 50 ml conical tube with plunger from a 10 ml syringe. Mashed spleens were rinsed 2 times and centrifuged at 2000 rpm for 5 min and resuspended in ACK lysing buffer for 5 min at room temperature. The splenocytes

were washed in PBS-gentamycin, resuspended in 5% RPMI and counted. Splenocytes were used for proliferation assay.

Antibody titers:

A/Vietnam/1203/2004 (H5N1); A/Anhui/1/05 (H5N1); A/turkey/Turkey/1/05 (H5N1); A/New Caledonia/20/99 (H1N2

[00170] Anti-influenza antibody titers of sera were measured at 14 days after the first immunization as well as 21 days after the second immunisation (at sacrifice). The titers were determined by enzyme-linked immunosorbent assay (ELISA) using the inactivated virus A/Indonesia/5/05 as the coating antigen. The end-point (titers were expressed as the reciprocal value of the highest dilution that reached an OD value of at least 0.1 higher than that of negative control samples.

[00171] For antibody class determination (IgG1, IgG2a, IgG2b, IgG3, IgM), the titers were evaluated on final bleeding by ELISA as previously described.

Hemagglutination inhibition (HI) titers

[00172] Hemagglutination inhibition (HI) titers of sera were measured at days 14 and 35 after the second immunisation as previously described (WHO 2002; Kendal 1982). Inactivated virus preparations from strains A/Indonesia/5/05; A/Anhui/1/05 (H5N1); A/turkey/Turkey/1/05 (H5N1) or A/Vietnam/1203/2004 were used to test rat 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% horse red blood cells. HI antibody titres were defined as the reciprocal of the highest dilution causing complete inhibition of agglutination.

Results

[00173] The reactivity of the sera from rats immunized with either the wt VLP or the mutant VLP was assessed 14 days after the first (Day 14) or the second immunization (Day 35). All rats were immunized with 15 μg of the antigen formulated with alum. Immunoreactivity was assessed against H5N1 viruses of clade 1 (A/Vietnam/1203/04),

clade 2.1 (A/Indonesia/5/05), clade 2.2 (A/turkey/Turkey/1/05) and clade 2.3 (A/Anhui/1/05). After the first dose, the mutant VLP induced a higher antibody reaction than the wt for all H5N1 strains tested (**Figure 6**). The immunoreactivity against the avian strain A/turkey/Turkey/1/05 was statistically significant ($p<0.05$) after the first dose. Immunoreactivity was also assessed against H1N1 viruses (A/New Caledonia/20/99) showing immunoreactivity after boost injection. GMT: geometric mean titer. Values are the GMT (ln) of reciprocal end-point titers of five rats per group. Bars represent mean deviation. * $p< 0.05$ compared to the wt VLP

[00174] HI titers from rats immunized with the wt or the mutant VLP were assessed 14 days after the first (Day 14) or the second (Day 35) immunization. HI antibody responses were measured using inactivated whole H5N1 viruses. After the first immunization, the mutant VLP induces a higher HI antibody response than the wt VLP against all H5N1 viruses tested (**Figure 7**). Statistical significance was reached for A/Indonesia/5/05 and A/turkey/Turkey/1/05 influenza strains. GMT: geometric mean titer. Values are the GMT (ln) of reciprocal end-point titers of five rats per group. Bars represent mean deviation. * $p< 0.05$ and compared to wt VLP.

[00175] These data strongly suggest that the "mutated" unglycosylated H5 protein represents a very interesting alternative to the native H5 protein for the production of VLPs as broad-spectrum and fast-active flu vaccine.

[00176] All citations are hereby incorporated by reference.

[00177] The present invention has been described with regard to one or more particular 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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Claims

1. An influenza virus hemagglutinin (HA) molecule having a HA1 domain, wherein said HA1 domain is totally or partially free of N-linked glycosylation.

2. The influenza virus hemagglutinin (HA) molecule as defined in claim 1, wherein one or more of N-linked glycosylation sites from HA1 domain is abolished, whereby the glycosylation site that is abolished is originally present on an immunogenic globular head portion of the protein.

3. The influenza virus hemagglutinin (HA) molecule as defined in claim 2, wherein said abolished glycosylation site is within or near a receptor binding subdomain of said immunogenic globular head or within or near an F'2 subdomain of the HA1 domain.

4. The HA molecule as defined in claim 3, wherein said glycosylation site is consisting of a glycosylation recognition triad N-X-S/T wherein N is asparagine, X is any amino acid except proline, S is serine and T is threonine.

5. The HA molecule as defined in claim 4, wherein one or more asparagine residue comprised in a glycosylation recognition triad N-X-S/T is substituted for a non-asparagine amino acid.

6. The HA molecule according to claim 5, wherein said asparagine residue is substituted for an amino acid selected from the group consisting of: Leu, Ile, Val, Thr, Ser and Ala.

7. The HA molecule according to claim 6, wherein said asparagine residue is substituted for an alanine.

8. The HA molecule as defined in claim 4 or 5 wherein one or more serine or threonine residue comprised in a glycosylation recognition triad N-X-S/T is substituted for a non-serine and non-threonine amino acid.

9. The HA molecule as defined in claim 8, wherein one or more serine or threonine residue comprised in a glycosylation recognition triad N-X-S/T is substituted for an amino acid selected from the group consisting of: Ala, Val, Ile and Leu.

10. The HA molecule according to claim 9, wherein said serine or threonine residue is substituted for an alanine.

11. The HA molecule according to any one of claims 1 to 10, wherein said glycosylation site is at a position corresponding to any triad located between amino acids 39 to 331 wherein numbering is in accordance with strain A/Vietnam/1194/04, whereby said abolished N-glycosylation site makes the HA molecule more antigenic with respect to antibodies specific for a native HA molecule.

12. The HA molecule according to any one of claims 11, wherein said glycosylation site is at a position selected from the group consisting of: 154, 165 and 286.

13. The HA molecule according to claim 12, having one glycosylation site abolished.

14. The HA molecule according to claim 12, having two glycosylation sites abolished.

15. The HA molecule according to claim 12, having three glycosylation sites abolished.

16. The influenza hemagglutinin (HA) according to any one of claims 1 to 15, wherein said influenza is of type A and type B.

17. The influenza hemagglutinin (HA) according to claim 16, wherein said influenza is of type B.

18. The influenza hemagglutinin (HA) according to claim 16, wherein said HA is from one or more than one 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.

19. The influenza hemagglutinin (HA) according to claim 18, wherein said HA subtype is one or more than one of subtype selected from the group consisting of: H1, H2, H3, H5, H7, H9, and H10.

20. The influenza hemagglutinin (HA) according to claim 19, wherein said HA subtype is one or more than one of subtype selected from the group consisting of: H1, H3, H5 and H7.

21. The influenza hemagglutinin (HA) of claim 16, wherein the influenza hemagglutinin is of type B or of type A subtype H3.

22. A nucleic acid comprising a nucleotide sequence encoding an influenza HA molecule according to anyone of claims 1 to 21.

23. A nucleic acid as defined according to SEQ ID NO. 17, wherein one or more residue encoding amino acids selected from the group consisting of: 154, 165 and 286 is encoding a non-asparagine.

24. The nucleic acid as defined in claim 23, wherein said one or more residue encoding amino acids selected from the group consisting of: 154, 165, and 286 is encoding alanine.

25. A nucleic acid as defined according to SEQ ID NO. 17, wherein one or more residue encoding amino acids selected from the group consisting of: 154+2, 165+2 and 286+2 is encoding a non-serine and non-threonine.

26. The nucleic acid as defined in claim 25, wherein said one or more residue encoding amino acids selected from the group consisting of: 154+2, 165+2, and 286+2 is encoding alanine.

27. A nucleic acid having 90% identity to the nucleic acid as defined in claim 23, 24, 25 or 26.

28. A nucleic acid as defined according to SEQ ID NO. 29.

29. A nucleic acid having 90% identity to SEQ ID NO. 29.

30. An expression cassette comprising a nucleic acid according to any one of claims 22 to 29, operatively linked to a regulatory region active in a non-sialylating host organism.

31. The expression cassette according to claim 30, wherein said regulatory region is selected from the group consisting of : plastocyanin regulatory region; napin promoter, the cruciferin promoter, or a regulatory region obtained from Ribulose 1,5-bisphosphate carboxylase/oxygenase (RuBisCO), chlorophyll a/b binding protein, ST-LS, polyhedron promoter, and the gp64 promoter.

32. The expression cassette according to claim 31, wherein said plastocyanin regulatory region is from alfalfa (*Medicago sativa*).

33. A method of producing influenza virus like particles (VLPs) in a non-sialylating host organism, said method comprising:

a) introducing a nucleic acid according to any one of claims 22 to 29, operatively linked to a regulatory region active in a non-sialylating host organism or a portion thereof, and

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

34. The method according to claim 33, wherein, in the step of introducing (step a), the nucleic acid may be either transiently expressed in the host, or stably expressed in the host.

35. The method according to claim 33 or 34, wherein said non-sialylating host organism is selected from the group consisting of: plant, insect and yeast.

36. The method according to claim 35, wherein said plant is selected from the group consisting of: a plant cell, a whole plant and a portion thereof.

37. The nucleic acid according to claim 36, wherein said plant cell is from *N. benthiamina.*

38. The method of claim 33 further comprising a step of : c) harvesting the host and purifying the VLPs.

39. A virus like particle (VLP) comprising an influenza hemagglutinin molecule having a HA1 domain, wherein one or more of the N-linked glycosylation site from said HA1 subdomain has been abolished.

40. The VLP according to claim 39, wherein said VLP is immunogenic.

41. A virus like particle (VLP) encoded by the nucleic acid according to any one of claims 22 to 29.

42. A virus like particle (VLP) comprising an influenza virus HA protein according to any one of claims 1 to 21, and one or more than one host lipid.

43. The virus-like particle according to claim 41 or 42, wherein said host lipid is a plant lipid.

44. A virus like particle (VLP) according to claims 41, 42 or 43, comprising an influenza virus HA bearing plant-specific N-glycans, or modified N-glycans.

45. The virus like particle (VLP) of claim 44 wherein the influenza HA protein is H5 Indonesia.

46. Use of the VLP according to any one of claims 39 to 45 for the manufacture of a vaccine for the prevention or treatment of a viral infection in a subject.

47. Use according to claim 46, wherein said subject is selected from the group consisting of: human or birds.

48. Use of the VLP according to any one of claims 39 to 45 for the prevention or treatment of an influenza viral infection in a subject.

49. Use according to claim 48, wherein said subject is selected from the group consisting of: human or birds.

50. A composition comprising an effective dose of a VLP according to any one of claims 39 to 45, in admixture with a pharmaceutically acceptable carrier.

51. The composition according to claim 50, wherein said pharmaceutically acceptable carrier is suitable for oral, intradermal, intranasal, intramuscular, intraperitoneal, intravenous, or subcutaneous administration.

52. A composition for immunizing an individual at risk of contracting an influenza viral disease, said composition comprising: a VLP according to any one of claims 39 to 45 in admixture with a pharmaceutically acceptable carrier.

53. The composition according to claim 52 wherein said composition is in liquid, suspension, solid or powder form.

54. The composition according to claim 53 wherein said composition is suitable for oral, intradermal, intranasal, intramuscularly, intraperitoneal, intravenous, or subcutaneous administration.

55. A vaccine composition comprising an immumologically effective does of a VLP according to any one of claims 39 to 45, in admixture with a pharmaceutically acceptable carrier with or without the presence of an adjuvant.

56. The vaccine according to claim 55, wherein said pharmaceutically acceptable carrier is suitable to be administered orally, intradermally, intranasally, intramuscularly, intraperitoneally, intravenously, or subcutaneously.

57. The vaccine according to claim 56, devoid of said adjuvant.

58. A viral vaccine comprising: a VLP according to any one of claims 39 to 45 in admixture in a physiologically acceptable excipient or adjuvant.

59. The vaccine according to claim 58, being a human or avian vaccine.

60. The vaccine according to claim 59, being suitable for prophylaxis prior to appearance of symptoms; or for treatment of infection after appearance of symptoms

61. 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 according to any one of claim 1 to 21, or encoded by the nucleic acid according to any one of claims 22 to 29, one or more than one host lipid, and a pharmaceutically acceptable carrier.

62. The method according to claim 61, wherein said virus-like particle is administered to a subject orally, intradermally, intranasally, intramuscularly, intraperitoneally, intravenously, or subcutaneously.

63. A method for inducing immunity to influenza virus infection in a subject comprising administering an effective dose of a composition according to claim 50, or a vaccine according to claim 55.

64. A method of inducing immunity to an influenza virus infection in a subject, comprising administering the virus-like particle according to any one of claims 39 to 45.

65. The method of claim 64, wherein the virus like particle is administered to a subject orally, intradermally, intranasally, intramuscularly, intraperitoneally, intravenously, or subcutaneously.

66. The method according to any one of claims 61 to 65, wherein said subject 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.

67. The method according to claim 66, wherein the subject is selected from the group consisting of: human and birds.

68. The method according to claim 67, wherein the subject is human.

69. A container comprising the vaccine according to claim 55.

70. The container according to claim 69, said container containing a single unit dose.

71. The container according to claim 69, said container being a in multiple dosage

form.

72. The container according to claim 69, 70 or 71, said container being a syringe.

73. A syringe pre-filled with the vaccine according to claim 55.

74. A kit comprising a container according to any one of claims 69 to 73, and instructions on how to use/administer said vaccine.

75. A plant cell transformed with a nucleic acid according to any one of claims 22 to 29.

76. The plant cell according to claim 75, being transiently or stably transformed.

77. A portion of a plant transformed with a nucleic acid according to any one of claims 22 to 29.

78. The portion of a plant according to claim 77, being transiently or stably transformed.

79. A plant transformed with a nucleic acid according to any one of claims 22 to 29.

80. The plant according to claim 79 being transiently or stably transformed.

81. An *Agrobacterium* cell transfected with an expression cassette according to claim 30, 31 or 32.