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Biochemical composition of haemagglutinin-based influenza virus-like particle vaccine produced by transient expression in tobacco plants

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Summary

Influenza virus-like particles (VLPs) are noninfectious particles resembling the influenza virus representing a promising vaccine alternative to inactivated influenza virions as antigens. Medicago inc. has developed a plant-based VLP manufacturing platform allowing the large-scale production of GMP-grade influenza VLPs. In this article, we report on the biochemical compositions of these plant-based influenza candidate vaccines, more particularly the characterization of the *N*-glycan profiles of the viral haemagglutinins H1 and H5 proteins as well as the tobacco-derived lipid content and residual impurities. Mass spectrometry analyses showed that all *N*-glycosylation sites of the extracellular domain of the recombinant haemagglutinins carry plant-specific complex-type *N*-glycans having core α(1,3)-fucose, core β(1,2)-xylose epitopes and Lewis^a extensions. Previous phases I and II clinical studies have demonstrated that no hypersensibility nor induction of IgG or IgE directed against these glycans was observed. In addition, this article showed that the plant-made influenza vaccines are highly pure VLPs preparations while detecting no protein contaminants coming either from *Agrobacterium* or from the enzymes used for the enzyme-assisted extraction process. In contrast, VLPs contain few host cell proteins and glucosylceramides associated with plant lipid rafts. Identification of such raft markers, together with the type of host cell impurity identified, confirmed that the mechanism of VLP formation *in planta* is similar to the natural process of influenza virus assembly in mammals.

Introduction

The recent swine H1N1 influenza pandemic revealed the limitations of the current influenza vaccine manufacturing technologies and has shed light on the need for new efficient influenza vaccines and manufacturing practices. Influenza virus-like particles (VLPs) are noninfectious particles resembling the influenza virus. These VLPs represent a promising alternative to inactivated influenza virions as antigens, and they have shown uniqueness by inducing potent immune responses (Roldão *et al.*, 2010). Medicago inc. has developed a plant-based VLP manufacturing platform based on the transient *Agrobacterium*-mediated expression of haemagglutinin proteins in tobacco and capable of producing influenza VLPs with unprecedented speed (D'Aoust *et al.*, 2008 and D'Aoust *et al.*, 2010). In this platform, influenza VLP expression and purification technologies were brought to large-scale production of GMP-grade material. As the influenza virus, plant-made influenza VLPs has a lipid envelop acquired during the particle budding process but only contain its haemagglutinin immunogenic determinants (Vézina *et al.*, 2011). Transmission electron microscopy imaging of tobacco leaves expressing VLPs indicated that the candidate vaccines accumulate out of the cell between the plasma membrane and the cell wall (D'Aoust *et al.*, 2008; Vézina *et al.*, 2011).

An initial publication reported that transient expression of the haemagglutinin (HA) protein could generate large numbers of H5-VLPs that were highly immunogenic in mice (D'Aoust *et al.*, 2008). Then, it was demonstrated that the first doses of a plant-made VLP candidate vaccine can be produced within 3 weeks after identification of a new pandemic strain (D'Aoust *et al.*, 2010). We finally reported on safety and immunogenicity from a phase I clinical study of the plant-made H5-VLP vaccine in healthy adults (Landry *et al.*, 2010; Ward *et al.*, 2014).

Haemagglutinin is a surface protein composed of a globular head, a stem and a cytosolic tail region. Numerous *N*-glycosylation sites are located on its globular head and stem region. The glycosylation of these sites is thought to affect the immunogenicity of an influenza strain by masking antigenic regions and

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proteolytic cleavage site of the protein (Abe *et al.*, 2004; Munk *et al.*, 1992) or by decreasing receptor binding affinity (Gambaryan *et al.*, 1998; Matrosovich *et al.*, 1999). As a consequence, the expression of HA in a heterologous expression system raises the question of the *N*-glycosylation introduced on the recombinant vaccine. The *N*-glycosylation of secreted proteins in higher organisms is conserved but differs slightly in detail. Indeed, the expression of recombinant proteins in plants results in the production of bio-molecules exhibiting a plant-specific glycosylation. Main feature of *N*-glycosylation of plant proteins is the absence of sialic acids (Séveno *et al.*, 2004) and the presence on the core *N*-glycan of carbohydrate motifs that are not found in mammals, such as a core β(1,2)-xylose and a core α(1,3)-fucose residues (Lerouge *et al.*, 1998). These plant glyco-epitopes are of major interest in the context of the production of proteins dedicated to human therapy because they could be immunogenic in mammals (Bardor *et al.*, 2003) and could be involved in allergic reactions (Altmann, 2007). Additionally, the production in tobacco of VLP vaccines used for clinical studies addresses the question of putative co-purification of residual impurities arising from the *Agrobacterium* strains used for transient expression of HA or from enzyme used for the extraction process.

In this article, we report on the site-specific *N*-glycosylation of haemagglutinin from either A/New Caledonia/7/2009 (H1N1) or A/Indonesia/5/05 (H5N1) strains expressed in *Nicotiana benthamiana* and accumulated as plant-based VLPs. Moreover, the presence in H1-VLP and H5-VLP vaccines of host cell bio-molecules or process-related contaminants was investigated.

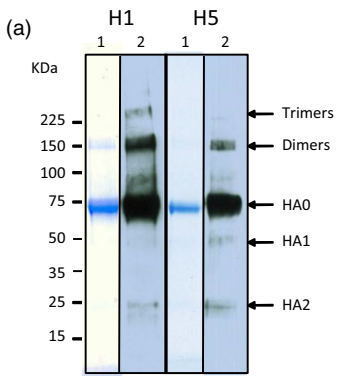
Results

Protein sequence analysis of haemagglutinins associated with tobacco-derived VLPs

Native protein sequence coding for the HA from strain A/New Caledonia/7/2009 (H1N1) or A/Indonesia/5/05 (H5N1) was expressed in *Nicotiana benthamiana* plants without the need of modifying the protein sequence. Tobacco-derived HA VLPs were isolated from leaves by enzyme degradation of the cell wall and then by successive purification steps as essentially previously described (Landry *et al.*, 2010). Purified HA VLPs obtained from this manufacturing process show particle size ranging from 130 to 140 nm exhibiting a unique peak as indicated by dynamic light scattering analysis (Vézina *et al.*, 2011). A first evaluation of the protein composition of plant-made H1-VLP and H5-VLP vaccines was carried out by gel electrophoresis. The viral H1 and H5 proteins represent 98–99% of proteins in the VLP vaccines as evaluated by Coomassie blue-stained gel and densitometry (Figure 1a). All the bands detected by densitometry were further analysed by mass spectrometry to include in the purity calculation only bands that had very low contamination with proteins other than the HA protein. HA0, the full length haemagglutinin precursor sequence, is detected as the main protein band in both the SDS-PAGE and the immunoblots using specific polyclonal antibodies raised against haemagglutinin (Figure 1a). HA1 and HA2 bands and dimers of HA0 are also detected with low intensity. HA1 and HA2 fragments result from the cleavage of the precursor HA0 at Arg_{327} and Arg_{330} of the haemagglutinin proteolytic sites of H1 and H5, respectively, as indicated by the arrows in Figure 1b and c. N-terminal sequencing of H5 enabled the identification of $DQICIGYHAN_{10}X_{11}STEQV$ (X; nonidentified amino acid). N-terminal sequence demonstrating that the H5 signal peptide is properly cleaved in the tobacco cell (Figure 1c). Moreover, this analysis also showed that Asn_{11} of the $N_{10}NST_{13}$ sequence is the only glycosylation site used in the mature HA.

N-glycosylation of tobacco-derived H1 and H5

H1 and H5 haemagglutinins are both glycoproteins exhibiting seven potential *N* -glycosylation sites, with consensus sequence N-X-S/T. Six of them are located in the HA1 globular head or the HA2 stem regions, whereas the site located in the cytosolic tail



(b)

DTLCIGYHAN**N**[11]STDTVDTVLEK**N**[23]VTVTHSVNLLEDKHNGKLCKLRGVAPLHLGKCNIAGWILGNPECESLSTASSWSYIVETPSSD**N**[87]G
TCYPGDFIDYEELREQLSSVSSFERFEIFPKTSSWPNHDSNKGVTAACPHAGAKSFYKNLIWLVKKGNSYPKLSKSYINDKGKEVLVLWGIHH
PSTSADQQSLYQNADAYVFVGSSRYSKKFKPEIAIRPKVRDQEGRMNYYWTLVEPGDKITFEATGNLVVPRYAFAMERNAGSGIIISDTPVH
DC**N**[275]TTCQTPKGAI**N**[287]TSLPFQNIHPITIGKCPKYVKSTKLRLATGLRNIPSIQSR↕GLFGAIAGFIEGGWTGMVDGWYGYHHQNEQGS
GYAADLKSTQNIDEITNKVNSVIEKMNTQFTAVGKEFNHLEKRIENLNKKVDDGFLDIWTYNAELLVLLENERTLYHDSNVKNLYEKVRSQLK
NNAKEIGNGCFEFYHKCDNTCMESVK**N**[481]GTYDYPKYSEEAKLNREEIDGVKLESTRIYQILAIYSTVASSLVLVVSLGAISFWMCS**N**[540]GSLQ
CRICI

(c)

DQICIGYHAN**N**[11]**ST**EQVDTIMEK**N**[23]**VT**VTHAQDILEKTHNGKLCDLDGVKPLILRDCSVAGWLLGNPMCDEFINVPEWSYIVEKANPTND
LCYPGSFNDYEELKHLLSRINHFEKIQIIPKSSWSDHEASSGVSACPYLGSPSFFRNVVWLIKK**N**[154]**ST**YPTIKKSY**N**[165]**NT**NQEDLLVLWGIHH
PNDAAEQTRLYQNPTTYISIGTSTLNQRLVPKIATRSKVNGQSGRMEFFWTILKPNDAINFESNGNFIAPEYAYKIVKKGDSAIMKSELEYGN
CNTKCQTPMGAI**N**[286]**SS**MPFHNIHPLTIGECPKYVKSNRLVLATGLRNSPQRESRRKKR↕GLFGAIAGFIEGGWQGMVDGWYGYHHSN
EQGSGYAADKESTQKAIDGVTNKVNSIIDKMNTQFEAVGREFNNLERRIENLNKKMEDGFLDVWTYNAELLVLMENERTLDFHDSNVKNL
YDKVRLQLRDNAKELGNGCFEFYHKCDNECMESIR**N**[484]**GT**YNYPQYSEEARLKREEISGVKLESIGTYQILSIYSTVASSLALAIMMAGLSLW
MCS**N**[543]**GS**LQCRICI

Figure 1 (a) SDS-PAGE analysis of H1-VLP and H5-VLP vaccines. (1) Coomassie G-250 stained SDS-PAGE of H1-VLP (2.5 μg) and H5-VLP (5 μg) vaccines under reducing conditions. (2) Western blot analysis of H1-VLP (0.5 μg) and H5-VLP (4 μg) vaccines using polyclonal antibodies raised against haemagglutinin of H1N1 (strain A/New Caledonia/7/2009) and H5N1 (strain A/Indonesia/5/05). (b and c): Sequences of H1 and H5, respectively. HA0, HA1 and HA2 refer to full H1 and H5 sequences and subunits, respectively. The underlined N-terminal peptide sequence of H5 was confirmed by Edman degradation. *N*-glycosylation sites are indicated in bold. ↕ indicates the proteolytic cleavage site between HA1 and HA2 haemagglutinin fragments.

region is not glycosylated (Blake *et al.*, 2009). The overall *N*-glycan profiles of H1-VLP and H5-VLP vaccines have been previously reported by mass spectrometry analysis of *N*-linked glycans released from the two recombinant proteins (Ward *et al.*, 2014). In the two samples, the most abundant *N*-glycan corresponds to the complex-type *N*-glycan $Gn_2M_3XFGn_2$ which exhibits both core α(1,3)-fucose and β(1,2)-xylose glycan epitopes that are usually found on plant *N*-glycans (Lerouge *et al.*, 1998; Viëtor *et al.*, 2003) (Table 1). The other identified *N*-glycans are complex oligosaccharides lacking either fucose or terminal GlcNAc residues. Moreover, *N*-glycans carrying β(1,3)-galactose and α(1,4)-fucose attached to the terminal N-acetylglucosamine (Lewis[a] structure; Le[a]) have been also detected in H1 and H5. In addition to complex *N*-glycans, H1-VLP vaccine also carries hybrid structures GnM_4XGn_2 and GnM_4XFGn_2 (Ward *et al.*, 2014) (Table 1). Minor GnM_5XGn_2 and GnM_5XFGn_2 were also detected in H1 *N*-glycan profile. These *N*-glycans result from the partial removal of mannose residues linked to the α(1,6)-Man arm of the Man-5 *N*-glycan by Golgi α-mannosidase II (Lerouge *et al.*, 1998).

The site-specific distribution of glycans on the six *N*-glycosylation sites of H1 and H5 was investigated by analysis of haemagglutinin tryptic peptides by liquid chromatography coupled to a nano-electrospray ionization source (LC-ESI MS/MS). In the first experiment, LC-ESI MS/MS analysis of H1 and H5 tryptic peptides was carried out before and after deglycosylation treatment with peptide *N*-glycosidase A (PNGase A). This enzyme is able to cleave all types of *N*-linked glycans to plant proteins (Tretter *et al.*, 1991). After deglycosylation, Asn residues of the occupied *N*-glycosylation sites are converted into Asp which enabled the identification of glycosylated peptides by mass spectrometry (Table 2). LC-ESI MS/MS analysis of deglycosylated H5 allowed for the identification of aspartic-containing tryptic peptides corresponding to the six *N*-glycosylation sites, indicating that all putative *N*-glycosylation sites of the extracellular region and stem region of H5 are used. In the case of H1, only five of the six *N*-glycosylation sites were identified as deglycosylated peptides using this approach. Due to its large size, the Asn87-containing tryptic peptide was not detected by LC-ESI MS/MS after PNGase A deglycosylation. To overcome this technical problem, H1 was digested by the endoproteinase GluC and the resulting peptides were analysed by mass spectrometry. This allowed for the demonstration that the Asn87 also carries *N*-glycans in H1-VLP vaccine (Table 2). Moreover, these proteomic analyses led to the overall coverages of 71–74% of the H1 and H5 sequences, indicating that both plant-based H1-VLP and H5-VLP vaccines do not exhibit unexpected post-translational modifications on the detected protein sequences.

To determine the distribution of *N*-glycans on the six *N*-glycosylation sites of H1 and H5, ions detected in the ESI MS were assigned to their respective glycopeptides on the basis of the molecular weight (MW) of the tryptic peptides containing a glycosylation site and of MW of oligosaccharides detected in the overall *N*-glycan population (Table 1). Assignment of ions to glycopeptides was first confirmed by the presence of reporter ions at m/z 204 and m/z 366 which correspond, respectively, to the oxonium ions of GlcNAc residues and of hexose-GlcNAc disaccharides in the MS/MS spectra. These two reporter ions are specifically observed in the fragmentation patterns of *N*-linked glycopeptides (Blake *et al.*, 2009). The structure of *N*-glycans attached to Asn residues of H1 and H5 was confirmed by analysis of MS/MS fragmentation pattern of the glycopeptides. For instance, Figure 2 shows the MS/MS of a triply charged Asn23-containing glycopeptide found in H5 carrying a complex *N*-glycan with two Le[a] epitopes. In addition to ion corresponding to the doubled charged N^{23}-E^{34} peptide at m/z 735, ions corresponding to the peptide carrying one and two GlcNAc (m/z 836 and 937) were detected in the MS/MS spectrum confirming that this peptide is *N*-glycosylated. Other fragment ions resulted from cleavages of glycosidic bonds of the glycan moiety or to the m/z 366 reporter fragment. In addition, m/z 512 oxonium ion observed in the MS/MS spectrum confirmed the occurrence of a terminal Le[a] epitope on the H5 glycopeptide. Taking

Table 1 Structures of *N*-linked glycans identified on plant-derived H1-VLP and H5-VLP vaccines. ■: GlcNAc; ●: Man; ▲: Fuc; ○: Gal; ☆: Xyl (Symbol nomenclature according to Varki *et al.*, 2009). Le[a]: Lewis[a] epitope

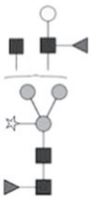
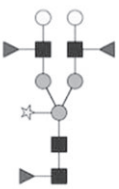
M_3XFGn_2	GnM_3XGn_2	GnM_3XFGn_2	GnM_4XGn_2	$Gn_2M_3XGn_2$
				
GnM_4XFGn_2	$Gn_2M_3XFGn_2$	$Le^aM_3XFGn_2$	$Le^aGnM_3XFGn_2$	$Le^a{}_2M_3XFGn_2$
				

Table 2 Site-specific distribution of *N*-linked glycans on H1 and H5 VLPs Figure

H1-VLP	N-glycans associated	H5-VLP	N-glycans associated
Asn11		Asn11	
Asn23		Asn23	
Asn87		Asn154	
Asn275		Asn165	
Asn287		Asn286	
Asn481		Asn484	

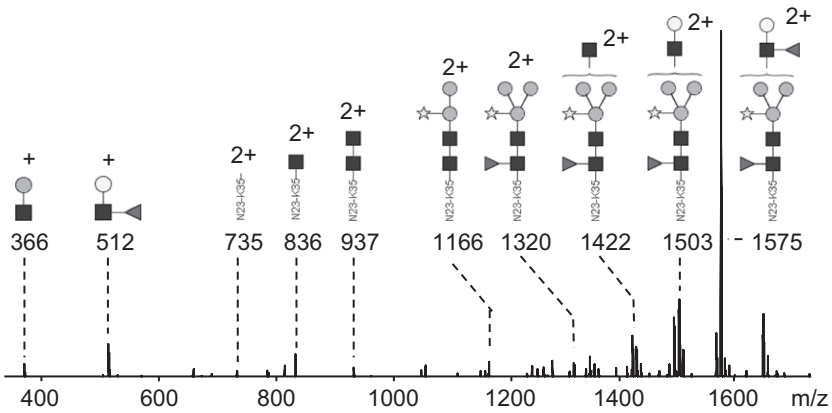


Figure 2 ESI MS/MS of triply charged H5 Asn23-containing peptide at m/z = 1221.5 carrying a $Le^a_2M_3XFGn_2$ *N*-glycan. ■: GlcNAc; ●: Man; ▲: Fuc; ○: Gal; ☆: Xyl (Symbol nomenclature according to Varki *et al.* (2009)).

together, these MS analyses showed that all *N*-glycosylation sites of H5 carry plant complex *N*-glycans and that the *N*-glycan distribution only slightly differs from one site to another (Table 2). The same conclusion can be drawn for the site-specific distribution of *N*-linked glycan on H1 (Table 2). However, as mentioned before, the *N*-linked glycans to Asn87 cannot be identified by tryptic digestion of H1 because the resulting Asn87-containing glycopeptide is too large to allow its analysis by mass spectrometry. As a consequence, the glycan distribution on Asn87 was investigated by digestion of H1 by the endoproteinase GluC and analysis of the glycopeptides by mass spectrometry (Table 2). In conclusion, this study demonstrated that the six *N*-glycosylation sites, located either in the HA1 globular heads or the HA2 stem regions of H1-VLP and H5-VLP vaccines, carry complex or hybrid glycans containing core α(1,3)-fucose or β(1,2)-xylose epitopes.

Analysis of host cell and excipient proteins in tobacco-derived influenza VLP vaccines

The presence of host cell tobacco proteins in H1-VLP and H5-VLP vaccines used for clinical studies was investigated by LC-ESI MS/MS. Table 3 lists all the proteins identified in both H1-VLP

Table 3 Proteins identified in H5-VLP and H1-VLP vaccines and proteins reported in lipid rafts according to Morel *et al.* (2006)

Proteins	H5-VLP	H1-VLP	Identified in *Nicotiana tabacum* lipid rafts
Influenza A virus, A/Indonesia/5/2005 (H5N1)	✓	Not detected	
Influenza A virus, A/California/07/2009 (H1N1)	Not detected	✓	
Plasma membrane ATPase 1 (*Nicotiana sp.*)	✓	✓	✓
Plasma membrane ATPase 4 (*Nicotiana sp.*)	✓	✓	✓
Ubiquitin (*Nicotiana sp.*)	✓	✓	Not detected
Harpin-inducing protein 1-like 18 (*Nicotiana sp.*)	✓	✓	✓
Pleiotropic drug resistance protein 1 (*Nicotiana sp.*)	✓	✓	Not detected
Carbonic anhydrase (*Nicotiana sp.*)	✓	✓	✓
Monosaccharide transporter (*Nicotiana sp.*)	✓	✓	✓
Syntaxin-related protein Nt-syr1 (*Nicotiana sp.*)	✓	✓	✓
Hypersensitive-induced response protein (*Arabidopsis sp.*)	✓	✓	✓
Water channel protein (*N. excelsior*)	✓	✓	✓
Ribulose bisphosphate carboxylase (RuBisCO) large chain (*N. debneyi*)	✓	✓	Not detected
NtElG-A1 protein (*Nicotiana sp.*)	✓	Not detected	Not detected
Heat-shock protein 70–3 (*Nicotiana sp.*)	✓	✓	✓
14-3-3 protein (*Nicotiana sp.*)	✓	✓	Not detected
Elongation factor (*Nicotiana sp.*)	✓	Not detected	✓
Molecular chaperone Hsp90 (*Nicotiana sp.*)	✓	Not detected	✓
Actin (*Nicotiana sp.*)	✓	Not detected	✓
Beta-tubulin (*Nicotiana sp.*)	✓	Not detected	✓
Ras-related protein RAB8 (*Nicotiana sp.*)	Not detected	✓	✓
Pto kinase interactor protein (*Nicotiana sp.*)	Not detected	✓	✓

and H5-VLP samples. As expected, the protein identified with the highest score is haemagglutinin. Interestingly, most of residual proteins found in H1-VLP and H5-VLP were previously identified in *Nicotiana tabacum* lipid rafts (Mongrand *et al.*, 2004; Morel *et al.*, 2006). For instance, plasma membrane ATPase was shown to be enriched in lipid raft as compared to plasma membrane (Mongrand *et al.*, 2004). The residual host cell protein profile found in H1 and H5 vaccines support the hypothesis that the HA recombinantly expressed in Medicago's platform accumulates and buds from plasma membrane lipid rafts in a mechanism, similar to mammalian host cell systems, that excludes the host plant proteins. Ribulose bisphosphate carboxylase/oxygenase (RuBisCO), the most abundant protein in plants, was detected in trace amount both H1-VLP and H5-VLP samples, while it was not detectable on SDS-PAGE. According to data with regard to plant allergen families (Radauer *et al.*, 2008), no proteins with known allergenic potential were identified in the impurity list, even in trace amounts in the VLP vaccine preparations. Finally, no process-related contaminants, either from *Agrobacterium* or from the enzymes used for extraction, were identified.

Lipid and glycolipid composition of plant-made VLPs

The HA proteins acquire a lipid envelop upon budding from the plant cell; therefore, plant-derived lipids are a constituent of the influenza vaccine. Indeed, the protein-lipid ratio is ca. 0.12–0.13 in plant-made HA VLPs (Vézina *et al.*, 2011). To characterize the lipid found in the HA VLP preparations, lipids were extracted following the method described by Bligh and Dyer (1959) and identified by LC-MS/MS. Table 4 shows lipids that were identified in both H1- and H5-VLP samples. Phosphatidylethanolamines (PE), phosphatidylcholines (PC) and phosphatidylserines (PS) were identified in all vaccine analysed with alkyl chains of different length (16 or 18 carbons) and number of unsaturations (0–3 double bonds). The LC-MS/MS method is not quantitative but, based on intensity of multiple reaction monitoring (MRM) signal, the most abundant structures in each class of lipids can be identified (Table 4, in bold). The presence of a glucosylceramide d18:2, h16:0 was also detected in the two vaccines (Table 4). According to Mongrand *et al.* (2004), glucosylceramide d18:2, h16:0 is the major polar lipid found in *N. tabacum* lipid rafts. This lipid was identified as the most abundant sphingolipid in all VLP samples. Other glucosylceramides and hydroxyceramides were also identified but at lower intensity.

Sugar composition of H5-VLP vaccine

VLPs carrying haemagglutinins are known to bud from the tobacco plasma membrane and are trapped by the primary cell walls of the host cells (D'Aoust *et al.*, 2008). In the extraction process of these vaccines, the tobacco cell walls are digested by polysaccharide-degrading enzymes which results in the release of large amounts of polysaccharide fragments. Analysis of residual sugar of the plant-made H5-VLP vaccine preparation was carried out by gas chromatography (GC) analysis. As expected, this analysis revealed the presence of Man, Xyl, Gal and Fuc monosaccharides arising from *N*-linked glycans (Tables 1 and 5). Glucose detected in the GC profile likely arose from glucosylceramides identified in the analysis of the VLP lipid fraction (Table 4). GalA and Rha were not detected demonstrating that VLP vaccine preparations are not contaminated by residual fragment of pectins, the acidic polysaccharide family of plant cell walls (Table 5).

Discussion

Plant-based production of recombinant therapeutics for human use is gaining more and more attention as products that achieve late-stage clinical development. One product even received FDA

Table 4 Phospholipids and sphingolipids identified in H1-VLP and H5-VLP vaccines. First column shows the structure of major phospholipids and sphingolipids. For each type of lipids, the lists of structures identified in H1-VLP and H5-VLP are listed. Most abundant structures are in bold

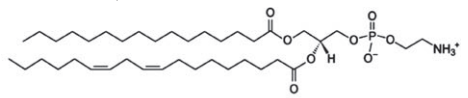
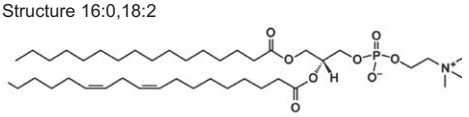
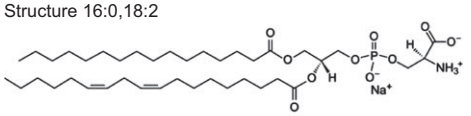
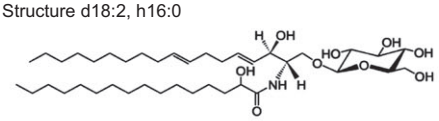
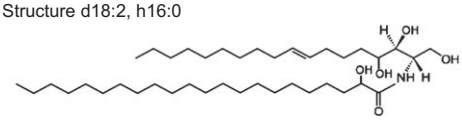
Lipids	H1-VLP	H5-VLP
Phosphatidylethalolamine (PE) Structure 16:0,18:2 	16:0,18:1 **16:0,18:2** **16:0,18:3** 18:0,18:0 18:0,18:1 **18:0,18:2** **18:1,18:2** **18:2,18:2** **18:3,18:2** 18:3,18:3 18:1, 20:0 18:1, 20:1 18:1, 20:2 18:1, 20:3	16:0,18:1 **16:0,18:2** **16:0,18:3** 18:0,18:0 18:0,18:1 **18:0,18:2** **18:1,18:2** **18:2,18:2** **18:3,18:2** 18:3,18:3 18:1, 20:0 18:1, 20:1 18:1, 20:2 18:1, 20:3
Phosphatidylcholine (PC) Structure 16:0,18:2 	**16:0,18:1** **16:0,18:2** **16:0,18:3** 18:0,18:0 18:0,18:1 **18:0,18:2** **18:1,18:2** **18:2,18:2** **18:3,18:2** **18:3,18:3** 18:1, 20:0 18:1, 20:1 18:1, 20:2 18:1, 20:3	**16:0,18:1** **16:0,18:2** **16:0,18:3** 18:0,18:0 **18:0,18:1** **18:0,18:2** **18:1,18:2** **18:2,18:2** **18:3,18:2** 18:3,18:3 18:1, 20:0 18:1, 20:1 18:1, 20:2 18:1, 20:3
Phosphatidylserine (PS) Structure 16:0,18:2 	16:0,18:1 16:0,18:2 **16:0,18:3** 18:0,18:0 18:0,18:1 **18:0,18:2** **18:1,18:2** 18:2,18:2 18:3,18:2 18:3,18:3 18:1, 20:0 **18:1, 20:1** **18:1, 20:2** 18:1, 20:3	16:0,18:1 16:0,18:2 **16:0,18:3** 18:0,18:1 **18:0,18:2** **18:1,18:2** 18:2,18:2 18:3,18:2 18:3,18:3 18:1, 20:0 **18:1, 20:1** **18:1, 20:2** 18:1, 20:3
Lipids	H1-VLP	H5-VLP
Glucosylceramide Structure d18:2, h16:0 	**d18:2, h16:0** d18:1, h16:0 d18:2, h18:0 d18:2, h20:0 d18:2, h22:0 d18:2, h24:1 **d18:2, h24:0** t18:1, h24:0 d18:2, h26:0 t18:1, h26:0	**d18:2, h16:0** d18:1, h16:0 d18:2, h18:0 d18:2, h22:0 t18:1, h22:0 d18:2, h24:1 **d18:2, h24:0** t18:1, h24:0 d18:2, h26:0 t18:1, h26:0
Ceramide and Hydroxyceramide Structure d18:2, h16:0 	t18:0, c16:0 t18:1, c16:0 t18:0, h16:0 t18:0, c20:0 t18:0, h20:0 t18:0, c22:0 t18:1, c22:0 t18:0, h22:0 t18:1, h22:0 d18:1, c24:1 t18:0, c24:0 t18:1, c24:0 t18:0, h24:0 t18:0, h24:1 t18:1, h24:0 t18:0, h26:0 t18:1, h26:0	t18:0, c16:0 t18:0, h16:0 t18:0, c20:0 t18:0, h20:0 t18:1, h20:0 t18:0, c22:0 t18:1, c22:0 t18:0, h22:0 t18:1, h22:0 d18:1, c24:1 t18:0, c24:0 t18:1, c24:0 t18:0, h24:0 t18:0, h24:1 t18:1, h24:0 t18:0, h26:0 t18:1, h26:0

Table 5 Monosaccharide composition of H5-VLP vaccine performed by gas chromatography analysis

Monosaccharide	Relative proportion
Ara	18 ± 4
Rha	n.d.
Xyl	4 ± 1
GalA	n.d.
Fuc	3 ± 1
Gal	33 ± 8
Man	25 ± 5
Glc	12 ± 4

n.d., not detected.

approval in 2012 for the treatment of Gaucher's disease (Protalix™). The importance of characterization of plant-made candidate vaccines, together with correlation product safety profile is of outmost significance. Data reported in this study showed that the viral H1 and H5 proteins represent an homogenous preparation made of ≥95% of proteins in the plant-derived VLP vaccines used for clinical studies. H1 and H5 are glycoproteins exhibiting seven *N*-glycosylation sites, six of them being located in the globular head or the stem regions, and therefore, their glycosylation acquired in the plant expression system was unknown. Recently, the glycosylation of plant recombinant H1 and H5 haemagglutinins was reported. However, these haemagglutinins were expressed in fusion to a KDEL retention signal and as a consequence, they accumulate in the ER and only carry oligomannosides (Shoji *et al.*, 2011; Zhang *et al.*, 2012). In this study, we report on the *N*-glycosylation of the plant-made haemagglutinins that are addressed to the plasma membrane and then anchored in the plant-made VLPs. The *N*-glycosylation of H1-VLP and H5-VLP vaccines investigated by mass spectrometry showed that all sites of the extracellular domain of recombinant haemagglutinins carry plant complex or hybrid *N*-glycans and that the glycan distribution only slightly differs from one site to another. Mainly complex mature *N*-glycans that are identified on H1 and H5 are typical plant-specific *N*-glycans carrying core α(1,3)-fucose, β(1,2)-xylose epitopes and Le^a extensions. The site-specific distribution of *N*-glycan on haemagglutinin of H1N1 and H5N1 strains grown in embryonated chicken eggs revealed that the six sites of the extracellular region are occupied by complex *N*-glycans (Blake *et al.*, 2009). As a consequence, we conclude that oligomerization and folding of H1 and H5 in the plant cell secretory system resemble those occurring in mammalian cells. The differences of *N*-glycan profiles between haemagglutinins from plant-derived VLP vaccines and H5N1 strain grown in chicken eggs only reflect the difference of Golgi glycosyltransferase repertoires between these two expression systems. No other post-translational modification of H1 and H5 was detected on peptides identified by proteomic analysis (sequence coverages are 71–74%).

Data reported in Landry *et al.* (2010) show that plant-made VLP vaccines are safe, well-tolerated and immunogenic. In this study, particular attention was given to the development of hypersensitive responses and to the production of antibodies against plant-specific glycan epitopes, such as core α(1,3)-fucose and core β(1,2)-xylose residues. No hypersensibility or induction of IgG or IgE directed against glycans was observed during the phase I clinical trial, thus demonstrating that immunization with HA VLPs did not trigger a response against plant glyco-epitopes (Landry *et al.*, 2010), although this study shows that such epitopes are widely distributed on haemagglutinins associated with plant-made VLP. Recently, subjects that received H1- or H5-VLP vaccines were monitored for 6 months. IgG and IgE to plant glyco-epitopes were measured by ELISA. No subject developed allergic/hypersensitivity symptoms. Some (34%) developed transient IgG and, in some cases IgE, to plant glyco-epitopes. Antibodies returned to baseline by 6 months in most subjects (Ward *et al.*, 2014).

The analysis of the sugar composition of H5-VLP vaccine indicated the absence of GalA in H5-VLP which supports the conclusion that the manufacturing process is efficient for eliminating pectin fragments released from the tobacco cell wall during the extraction process. The nature of residual proteins in drug substances was also investigated by LC-ESI MS/MS. Results showed that the plant-made VLPs are of high purity and no protein contaminants either from *Agrobacterium* or from the enzymes used for biomass digestion were identified. Moreover, no host cell proteins with known allergenic potential were identified in the impurity list, even in trace amounts, in the VLP vaccine preparations (Radauer *et al.*, 2008). This indicates that H1-VLP and H5-VLP vaccines used for clinical trials were free of any contaminant arising from the production and purification processes, which is consistent with the good safety profile obtained during the studies reported in Ward *et al.*, 2014.

VLPs rather contain few host cell proteins normally associated with plant lipid rafts (Mongrand *et al.*, 2004; Morel *et al.*, 2006). Moreover, glucosylceramide d18:2, h16:0 was also identified in all vaccines. This sphingolipid is the major polar lipid found in *N. tabacum* lipid rafts (Mongrand *et al.*, 2004). Identification of such lipid raft markers allowed the confirmation that the mechanism of VLP formation *in planta* is similar to the natural process of influenza virus assembly in mammalian host cells, implying the recruitment of the viral protein in the plasma membrane at lipid rafts, before budding out from the host cell when the appropriate local accumulation of haemagglutinin is reached.

Experimental procedures

Materials

Plant-made VLP vaccines bearing recombinant H1 or H5 from A/New Caledonia/7/2009 (H1N1) or A/Indonesia/5/05 (H5N1) strains were produced in *Nicotiana benthamiana* according to protocols reported in Landry *et al.*, 2010. Briefly, 6 days after infiltration of *Nicotiana benthamiana* with transgenic agrobacteria bearing the target HA gene, leaves were harvested and VLPs were purified by successive standard filtration and chromatographic steps.

SDS-PAGE electrophoresis and Western blots

Proteins from 0.5 to 5 μg of H1- and H5-VLPs were then separated on a 12% SDS-PAGE as reported in D'Aoust *et al.* (2008) and then revealed with Coomassie G-250. Haemagglutinins were immunodetected by Western blot analysis using polyclonal antibodies raised against H1 and H5.

Protein identification

Proteins were identified by LC-MS/MS using a NanoAcquity UPLC coupled to a QTOF micro through a nano-electrospray source (Waters). Following reduction (DTT) and alkylation

(iodoacetamide) of cysteines, proteins were digested overnight with trypsin (Sequencing grade; Promega, Madison, WI, USA) at a ratio of 1:20. Four μg of tryptic peptides is then separated on a ProteoPep III C18 1.8 μm, 150 μm ID × 100 mm IntegraFrit column (New Objective) using a water–acetonitrile gradient and analysed using data-dependant acquisition (DDA). Raw files are converted to pkl using ProteinLynx Global Server v2.1.5 (Waters). Proteins are identified using Mascot search engine v2.3 (Matrix Science, Boston, MA, USA) and UniProt databases. Only proteins identified with two peptides or more and *P*-value <0.05 are listed. Protein contents of eight lots of H1-VLP and 18 lots of H5-VLP vaccines were analysed.

Edman degradation

H5-VLP was separated on a 8% SDS-PAGE and then transferred to a PVDF membrane using the ProSorb system from Applied Biosystem. N-terminal sequence was then sequenced automatically by Edman degradation with the Procise P494 (Applied Biosystem, Foster City, CA, USA).

Site-specific distribution of glycans on N-glycosylation sites of haemagglutinins

H1- and H5-VLPs were digested following the protocol described previously on section 'Protein identification'. Glycopeptides were then analysed on two different LC-ESI MS/MS equipment: 6340 Ion Trap from Agilent Technologies and with NanoAcquity-QTOF micro from Waters. Data analysis was performed by two different ways. The first one was based on the presence of reporter ion m/z = 204 and 366, characteristic of *N*-glycan fragmentation; then *N*-glycopeptides were identified by assignation of the fragment ions. The second one was based on the action of PNGase A switching occupied Asn from consensus sequence to Asp. Linking data sets from glycosylated and deglycosylated peptides, *N*-glycans were attached to a specific glycosylation site. Complementary analysis following the same principle was performed with GluC instead of trypsin by Proteodynamics (Saint Beauzire, France).

Identification of sphingolipids and phospholipids by LC-MS/MS

Sphingolipids and phospholipids were identified by LC-MS/MS using a Surveyor-TSQ Quantum Ultra system (Thermo, Waltham, MA, USA). Lipids from four H1-VLP and seven H5-VLP vaccine lots were extracted following the method of Bligh and Dyer (1959). After evaporation of the organic layer, lipids were separated on a Hypersil BDS C8, 3 μm, 3 mm ID × 100 mm column (Thermo) using a water–methanol gradient, with ammonium formate and formic acid additives. Lipids were detected by multiple reaction monitoring (MRM) acquisition using Xcalibur 2.2 (Thermo). MRM transitions are listed for each class of lipids in Table S1.

Sugar composition analysis

Sugar composition of H5-VLP was determined by gas chromatography (GC) of monosaccharides released by acid hydrolysis. VLPs were hydrolysed with 2M TFA for 2 h at 110 °C. After cooling and neutralization, the sample was applied to a C18 reverse phase cartridge, and then, the monosaccharides were separated from membrane lipids by elution in water. The water fraction was freeze-dried and then submitted to a 16 h methanolysis at 80 °C with 500 μL of dry 1 M methanolic-HCl (Supelco). After evaporation of the methanol, the methyl glycosides were converted into their trimethylsilyl derivatives at 110 °C for 20 min with 200 μL of the silylation reagent (HMDS:TMCS:Pyridine, 3:1:9, Supelco) and then analysed by GC. The gas chromatograph (Varian CP-3800, Varian) is equipped with a flame ionization detector, a WCOT fused silica capillary column (length 25 m, i.d. 0.25 mm) with CP-Sil 5 CP as stationary phase and helium as gas vector. The oven temperature program was as follows: 2 min at 120 °C, 10 °C/min to 160 °C, and reach 220 °C by 1.5 °C/min prior to end the run by an increase of 20 °C/min to 280 °C. The quantification of sugar was carried out by the integration of peaks, and the determination of the corresponding molar values using response factors established with standard monosaccharides.

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Supporting information

Additional Supporting information may be found in the online version of this article:

Table S1 List of MRM transitions for lipids detection.

