ELPylated avian flu vaccines from plants: Improvement of expression and development of a new purification strategy

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List of abbreviations

Amino acid	Three letter code	One letter code	Amino acid	Three letter code	One letter code
alanine	Ala	A	leucine	Leu	L
arginine	Arg	R	lysine	Lys	K
asparagine	Asn	N	methionine	Met	M
aspartic acid	Asp	D	phenylalanine	Phe	F
cysteine	Cys	C	proline	Pro	P
glutamic acid	Glu	E	serine	Ser	S
glutamine	Gln	Q	threonine	Thr	T
glycine	Gly	G	tryptophan	Trp	W
histidine	His	H	tyrosine	Tyr	Y
isoleucine	Ile	I	valine	Val	V

Nucleotide code			
Adenine	A	Cytosine	C
Guanine	G	Thymine	T

ABA	Abscisic acid	BSA	Bovine serum albumin
° C	Celsius degree	*ca*	Cold-adapted phenotype
µg	Microgram	CaM V 35S	*Cauliflower mosaic virus* 35S promoter
4-MU	4-methylumbelliferone	Carb	Carbenicillin
A. thaliana	*Arabidopsis thaliana*	$Carb^R$	Carbenicillin resistance
aa	Amino acid	CD	Cluster of differentiation
AbISCO	AbISCO®-100 adjuvant	cDNA	Complementary DNA
Amp	Ampicillin	Cef	Cefotaxim
Amp^R	Ampicillin resistance	CFA	Complete Freund's adjuvant
APCs	Antigen presenting cells	cITC	Centrifugation-based ITC
att	Attenuated phenotype	CSL	The commonwealth serum laboratories
BN-PAGE	Blue native polyacrylamide gel electrophoresis	CT	Cytoplasmic tail
BS3	Bis[sulfosuccinimidyl] suberate	CTL	Cytotoxic T lymphocyte

cv.	cultivar	IB	Inclusion body
DCs	Dendritic cells	IFA	Incomplete Freund's adjuvant
DMSO	Dimethyl sulfoxide	Ig	Immunoglobulin
DNA	Deoxyribonucleic acid	IL	Interleukin
ECEs	Embryonated chicken's eggs	IMAC	Immobilized metal ion affinity chromatography
EID_{50}	50% Egg infectious dose	ISCOMs	Immunostimulating complexes
ELISA	Enzyme-linked immunosorbent assay	ITC	Inverse transition cycling
ELP	Elastin-like polypeptide	Kan	Kanamycin
EMEA	The European medicines evaluation agency	Kan^R	Kanamycin resistance
ER	Endoplasmic reticulum	Kan^S	Kanamycin sensitive
Et al	And co-workers	KD	Dissociation constant
etc	And others	KDa	Kilo dalton
FAO	Food and Agriculture Organization	LAIVs	Live-attenuated influenza vaccines
Fc	Fragment crystallizable	LD_{50}	50% Lethal dose
Fig.	Figure	LeB4 SP	Legumine B4 signal peptide
g	Gravity speed	LP	Low pathogenicity
g/L	Gram per liter	LPAI	Low pathogenic avian influenza
Gal	Galactose	M	Matrix protein
GSK	GlaxoSmithKline Biologicals	MDCK	Madin Darby canine kidney
h	Hour	MDV	Master donor viruse
H_2Odd	Dideionized water	MHC I&II	Major histocompatibility complex class I&II
HA or H	Hemagglutinin	MHLW	Ministry of health, labor and welfare (Japan)
HAU	Hemagglutination unit	min	minute
HBsAg	Hepatitis B virus surface antigen	mITC	Membrane-based ITC
HGR	High growth reassortant	mM	Millimolar
HI	Hemagglutination inhibition	NA or N	Neuraminidase
HIV-1	Human immunodeficiency virus HIV-1 type 1	Nb	*N. benthamiana*
HP	High pathogenicity	Ni-NTA	Nickel-nitrilotriacetic acid
HPAI	Highly pathogenic avian influenza	nm	Nanometer
HRP	Horseradish peroxidase	NP	Nucleoprotein

nptII	Gene coding neomycin Phosphotransferase II	SDS	Sodium dodecyl sulfate
NS	Nonstructure	Sec	Second
Nt	*N. tabacum*	SpaA	Surface protein antigen A of *Streptococus mutants*
OD	Optical density	T0	Primary transgene plant
OIE	World Organization for Animal Health	T1	Descendant of T0 plants
Oxa	2-phenyl-oxazol-5-one (oxazolone)	TBAG	Mycobacterial antigen
oxa	Oxazolone	$TCID_{50}$	50% Tissue culture infectious dose
PAGE	Polyacrylamide gel electrophoresis	Th1	T helper cell 1
PB	Protein body	Th2	T helper cell 2
PBS	Phosphate-buffered saline	TMD	Transmembrane domain
PCR	Polymerase chain reaction	TNFγ	Tumor necrosis factor γ
pII	Trimerization motif GCN4-pII	Trx	Thioredoxin
pLI	Tetramerization motif GCN4-pLI	*ts*	Temperature sensitive phenotype
pNPP	p-nitrophenyl phosphate	TSP	Total soluble protein
R	Resistant	U	Unit
RBCs	Red blood cells	USP	Unknown seed protein
RBD	Receptor binding domain	v/v	Volume per volume
rg	Reverse genetics	VLPs	Virus-like particles
Rif	Rifampicin	w/v	Weight per volume
RNA	Ribonucleic acid	WHO	World Health Oganization
rpm	Revolutions per minute	WT	Wild type
S	Sensitive	α	Alpha
SA	Sialic acid	β	Beta
SAP	Shrimp alkaline phosphatase	χ2	Chi-square
ScFv	Single chain variable fragment		

I. Introduction

I.1. Influenza A viruses

Influenza, commonly known as flu, is a highly infectious disease of birds and many mammals. It is caused by a single-stranded negative sense RNA virus belonging to the family *Orthomyxoviridae* (Swayne, 2008; Swayne and Suarez, 2000). Based on conserved proteins including nucleoproteins (NP) and matrix protein (M), the family is clustered into three different types: A, B and C. The influenza A and B viruses possess eight separate RNA segments, whereas the influenza C viruses contain seven RNA segments (Sfakianos, 2006). Influenza A viruses can be further classified into subtypes based on antigenic differences in the two surface glycoproteins: hemagglutinin (HA or H) and neuraminidase (NA or N) (Fouchier *et al.*, 2005). Another influenza surface protein is the matrix 2 protein (M2). This protein functions as an ion channel that plays an important role for triggering viral uncoating (Swayne, 2008). Other major viral proteins include the nucleoprotein (NP) which is the main structural protein, matrix 1 protein (M1), polymerase protein complexes which are the polymerase basic protein 1 (PB1), polymerase basic protein 2 (PB2), polymerase acidic protein (PA) and nonstructural proteins (NS1 and NS2) (Swayne, 2008) (Fig. I-1).

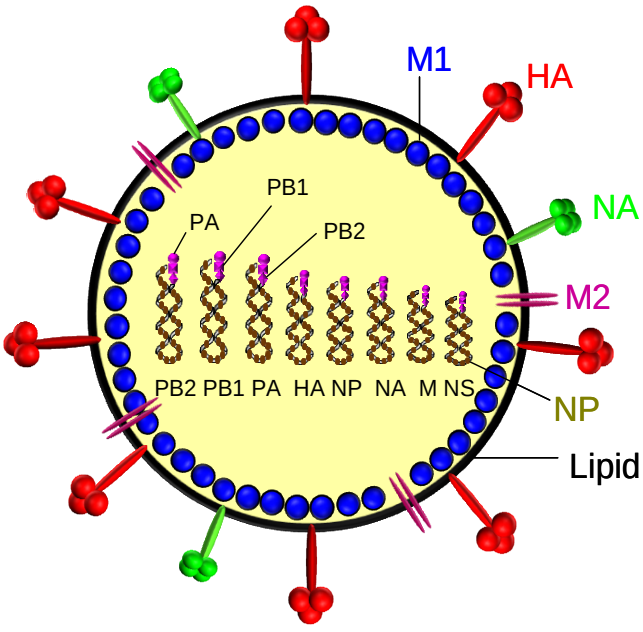

Figure I-1. Structure of influenza A virus. HA: hemagglutinin; NA: neuraminidase; M1 and M2: matrix proteins named as M1 and M2; NP: nucleoprotein; the polymerase complex including the polymerase basic protein 1 (PB1), polymerase basic protein 2 (PB2) and polymerase acidic protein (PA), NS: nonstructure proteins.

I.1.1. Hemagglutinin

The influenza virus contains two major surface glycoproteins: the hemagglutinin and neuraminidase which are integrated into the virus lipid membrane envelope. The influenza hemagglutinin plays important roles in (1) attaching to host cells by its binding to sialosaccharides located on the host cell surface and (2) facilitating membrane fusion between host cell and viral membrane *via* the fusion peptide located between HA1 and HA2 subunits (Harrison, 2008). Human influenza hemagglutinins prefer to bind sialic acid (SA)-α-2,6-Gal-terminated saccharides, whereas avian influenza hemagglutinins likely bind those terminating in SA-α-2,3-Gal (Baigent and McCauley, 2003; Mubareka and Palese, 2008).

The influenza HA protein is encoded by viral gene segment 4. Native hemagglutinin in the virion forms a non-covalently bound homotrimer. Each monomer is synthesized as a single polypeptide precursor (HA0) containing two subunits: HA1 which is responsible for formation of the receptor binding domain (RBD) at a globular head and a membrane proximal domain composed of HA2 which is responsible for membrane fusion (Fouchier *et al*., 2005; Gamblin and Skehel, 2010). Neutralizing antibodies targeting specifically to RBD of hemagglutinin protein can directly prevent the entry of influenza viruses into cells (Skehel and Wiley, 2000). Hemagglutinins contain a cleavage site at the HA1-HA2 junction. This cleavage is a determining factor for influenza virus pathogenicity. Low pathogenic (LP) viruses possess HA0 containing a single basic (arginine, R) residue (Q/E-X-R) at the cleavage site, while highly pathogenic (HP) viruses contain HA0 with multiple basic residues (QRERRRKKR). This motif is cleaved ubiquitously by not only trypsin-like proteases but also intracellular proteases like furin (Hulse *et al*., 2004; Klenk and Garten, 1994; Nayak *et al*., 2004).

When amino acid sequences of hemagglutinin are changed by point mutations, mutated hemagglutinin containing viruses are not recognized by antibodies which were induced by previous viruses. This progress is called an antigenic drift (DiMenna and Ertl, 2009; Swayne, 2008). Although the antigenic drift causes relatively small changes of the viral coding sequence, mutations are accumulated continuously. This is one main reason why influenza vaccines should be changed seasonally to fill the gap between circulating strains and vaccine strains. When genomic RNA segments encoding HA and NA dramatically changed by re-arrangements of those genomic segments between different influenza subtypes, new influenza subtype appears causing a pandemic. This big change in viral coding sequence is termed an antigenic shift (DiMenna and Ertl, 2009; Swayne, 2008).

Currently, sixteen subtypes of HA (H1-H16) and nine NA (N1-N9) antigenic variants are classified serologically in influenza A viruses (Fouchier *et al*., 2005). Of the 16 hemagglutinin subtypes, the H1, H2 and H3 viruses are known to circulate in human populations and caused the deadly pandemics in the past such as H1N1 in 1918 (Spanish pandemic), H2N2 in 1957 (Asian pandemic) and H3N2 in 1968 (Hong Kong pandemic) (Yen and Webster, 2009).

I.1.2. Neuraminidase

The neuraminidase of the influenza virus, a type II integral glycoprotein, is a surface glycoprotein and has enzymatic activity to remove α-2,3; α-2,6-linked sialic acid moieties from host or viral glycoproteins. The NA function prevents aggregation of virions due to the binding between HA and viral proteins and facilitates virus release (Sylte and Suarez, 2009; Veit and Thaa, 2011). Neuraminidase is a homotetramer consisting of four identical subunits and encoded by viral gene segment 6. The active site of the NA (each subunit of tetramer has one) is located on the top of globular domain containing a pocket of conserved amino acids: Arg 118, Arg 292, Arg 371, Arg 152 and Glu 276 (Russell *et al*., 2006). Of the 9 neuraminidase subtypes, only N1 and N2 neuraminidases of viruses currently circulate in human populations and clustered into two phylogenetically distinct groups (Thompson *et al*., 1994). N1, N4, N5 and N8 subtypes belong to group-1, whereas N2, N3, N6, N7 and N8 are group-2 (Russell *et al*., 2006).

Neuraminidase is an attractive target for the development of virus inhibitors because its active site is highly conserved in the influenza A and B viral strains and subtypes (Burmeister *et al*., 1992; Colman, 1994; Colman *et al*., 1983; Young *et al*., 2001). Currently, drugs such as Zanamivir (Relenza) (Colman, 2002) and Oseltamivir (Tamiflu) (Sweeny *et al*., 2000) are potent inhibitors of influenza neuraminidases and used to treat patients suffering from seasonal influenza viruses or pandemic influenza viruses (Monto, 2003; Moscona, 2005; Schunemann *et al*., 2007). Antibodies induced by neuraminidase protected mice fully that were vaccinated with two doses of 200 µg of N2 neuraminidase protein (Deroo *et al*., 1996), 100 µg of DNA expressing N1 neuraminidase (Sandbulte *et al*., 2007) from homologous virus challenges or protected partially (Sandbulte *et al*., 2007; Sylte *et al*., 2007) model animals from heterologous virus challenges. The number of neuraminidases presenting on the viral surface is much lower than those of hemagglutinins. To get balanced immune responses induced by hemagglutinin and neuraminidase, the exogenous neuraminidase was supplemented to conventional vaccine. This formulation reduced significantly replication of heterologous virus in comparison to the

non-supplemented vaccine (Johansson *et al*., 1998). Sandbulte and co-workers improved that antibodies which were induced by neuraminidase in seasonal vaccine (A/New Caledonia/20/99) could provide partial protection against H5N1 avian influenza infection (A/Vietnam/1203/04 (H5N1) in mice (Sandbulte *et al*., 2007).

I.1.3. Highly pathogenic avian H5N1 influenza virus

I.1.3.1. HPAI H5N1 virus as a potential pandemic threat

Of the 16 hemagglutinin subtypes, only H5 and H7 viruses are further classified into two different pathotypes: LP and HP in chickens and other gallinaceous birds (Yen and Webster, 2009). HP H5 was rarely observed to be lethal in wild birds. The only A/Tern/South Africa/61(H5N3) influenza virus caused the lethal deaths of aquatic birds was reported (Becker, 1967; Hulse-Post *et al*., 2005). However, HP H5N1 influenza viruses emerging in several Asian countries since the outbreak in Hong Kong in 2002 caused pathogenic deaths of wild birds, both aquatic and terrestrial poultry (Ellis *et al*., 2004). Hundreds of millions of chickens and ducks were died or culled with an effort to erase the HP H5N1 spread. The direct and indirect economic and tourism impact of HP H5N1 influenza viruses in Asian countries in 2003 and 2004 were more than 10 billion dollars (FAO, 2005). Although HP H5N1 influenza viruses are pathogenic to chickens and turkeys, they become less pathogenic to domestic ducks (Hulse-Post *et al*., 2005; Yen and Webster, 2009). Hulse-Post and co-workers proved that the some antigenic variants with diminishing pathogenicity were selected rapidly (only in single passage) in mallard ducks. It is suggested that waterfowl have exhibited higher resistance than chickens and other gallinaceous birds to H5N1 infection. They become hidden sources of HP H5N1 influenza viruses for maintenance and spread of the virus (Hulse-Post *et al*., 2005; Yen and Webster, 2009).

Basing on H5 sequence analysis, HP H5N1 influenza viruses were grouped into 10 distinct clades (numbered 0-9) by the H5N1 Evolution Working Group which was supported by three international agencies (the World Health Organization [WHO], the World Organization for Animal Health [OIE] and the Food and Agriculture Organization [FAO] (WHO/OIE/FAO H5N1 Evolution Working Group, 2008). The earliest HP H5N1 avian influenza virus, A/goose/Quangdong/96, emerged in 1997 was designated as clade 0 origin (Adams and Sandrock, 2010; WHO/OIE/FAO H5N1 Evolution Working Group, 2008; WHO, 2005). The HP H5N1 influenza viruses isolated in 2004 and 2005 from humans and birds in Thailand, Vietnam and Cambodia were distributed to clade 1. Meanwhile, viruses isolated from Indonesia, South Korea, Japan and China during the 2004-2005 outbreak were grouped

into clade 2 (Adams and Sandrock, 2010; WHO/OIE/FAO H5N1 Evolution Working Group, 2008; WHO, 2005; Yen and Webster, 2009). Clade 2 viruses continued to evolve into 5 additional second number order clades (subclades 2.1 to 2.5) (WHO/OIE/FAO H5N1 Evolution Working Group, 2008). Among these, the 2.1 viruses are responsible for Indonesian outbreaks (Adams and Sandrock, 2010). This subclade can be further separated into subclades 2.1.1, 2.1.2 and 2.1.3 (Yen and Webster, 2009). The subclade 2.2 viruses causing the pathogenic outbreak in Qinghai Lake in 2005 (the leading breeding site of migratory waterfowl) then extensively migrated to more than 30 countries in the Middle East, in Europe and in Africa (Adams and Sandrock, 2010; Chen *et al*., 2005; WHO/OIE/FAO H5N1 Evolution Working Group, 2008; WHO, 2005; Yen and Webster, 2009). Clade 2.3 viruses are predominantly circulated in China and currently spread to Vietnam, Thailand, Laos, Hong Kong and Malaysia. They were clustered further into 4 separate subclades 2.3.1, 2.3.2, 2.3.3 and 2.3.4 (Smith *et al*., 2006; Yen and Webster, 2009).

Since 2003, HP H5N1 avian influenza viruses have not only caused outbreaks in poultry but also transmitted to humans who contacted directly infected poultry with high mortality rates of 59% (332 deaths/566 human cases) (WHO, 2011). Moreover, H5N1 avian influenza viruses have become an increasing host range causing interspecies transmission among mammalian species such as cats (Rimmelzwaan *et al*., 2006; Weber *et al*., 2007), tigers and leopards in Thailand (Keawcharoen *et al*., 2004) and dogs (Songserm *et al*., 2006). In addition, the first recorded human case of HP H5N1 influenza virus transmission between family clusters was also confirmed (Ungchusak *et al*., 2005). These cases raise a concern that HP H5N1 virus poses as a highly potential pandemic threat virus in the future (Yen and Webster, 2009).

I.1.3.2. Development of vaccines for HP H5N1 influenza virus

Vaccination is the most effective approach to reduce illness and death from outbreaks of influenza viruses. Since hemagglutinin (Gerhard, 2001) and neuraminidase (Doherty *et al*., 2006) are the most important antigens for induction immunity, these glycoproteins are targets to develop influenza candidate vaccines. At present, two types of influenza vaccines are used to combat outbreaks of influenza viruses: inactivated influenza vaccine which is administrated intramuscularly and live attenuated influenza vaccine (LAIV) which is inoculated intranasally (Fiore *et al*., 2009).

I.1.3.2.1. Inactivated (whole, split and subunit) H5N1 influenza vaccines

In general, influenza vaccines are manufactured from influenza seed viruses that grow well in embryonated chicken's eggs (ECE). Seed viruses are generated by WHO Collaborating Centres and distributed to the vaccine manufacturers (Hickling and D'Hondt, 2006). Influenza vaccine viruses are harvested from allantoic fluid after 3 days propagated in ECE (Miyaki *et al*., 2010). Viruses are purified by ultracentrifugation on a sucrose gradient, then inactivated by chemicals using either formalin or β-propiolactone. Content of HA is determined and validated before whole viruses are used as vaccines. Additional filtration could be required to remove egg debris and bacterial contamination (Fiore *et al*., 2009; Hickling and D'Hondt, 2006). To reduce reactogenicity, the inactivated complete influenza viruses were disrupted using detergents such as triton X-100, sodium lauryl or Tween 80 (Hickling and D'Hondt, 2006; Roberts, 2008). The sucrose gradient ultracentrifugation is applied once more to separate the HA fraction from other fractions. Only HA-rich fractions were collected. The preparation is used as a vaccine and termed as a 'subvirion' or 'split' vaccine after detergents are removed by diafiltration (Hickling and D'Hondt, 2006). When HA in the second sucrose gradient purification is further purified to minimize contamination of NA, NP and other viral proteins, lipids; subunit or surface antigen vaccine is designated (Hickling and D'Hondt, 2006).

Because field influenza viruses are less efficient to grow in ECE, they are not directly used to produce influenza vaccines. Therefore, seed viruses with high growth property are required. The seed viruses are generated by genetic reassortment using field strains recommended by WHO and a high growth virus strain (A/Puerto Rico/8/34 (H1N1; PR8) which is adapted to grow well in ECE. A high growth reassortant (HGR) containing the antigenic components (HA, NA) of the WHO recommended strain and high growth properties of PR8 strain are selected (Kilbourne, 1969). With egg-based vaccine production, the time required for whole vaccine preparing process (from the identification of virus strains to ready-use/final vaccines) is up to 5-6 months (Kang *et al*., 2009; Wright, 2008).

Efforts to develop H5N1 influenza vaccines based on traditional approaches have been faced to several challenges. The HP H5N1 influenza viruses are highly pathogenic, so that embryonated chicken eggs are killed rapidly resulting in low viral titers (DiMenna and Ertl, 2009; El Sahly and Keitel, 2008). In initial studies, vaccines produced from LP A/Duck/Potsdam/1402-6/86 (H5N2) (Desheva *et al*., 2006) or LP A/Duck/Singapore/97 (H5N3) (Nicholson *et al*., 2001; Stephenson *et al*., 2005) influenza viruses were used to prevent the outbreak of HP H5N1 influenza viruses. The results of these studies showed that

although LP H5-based vaccines provided protective antibodies to HP H5N1 viruses, these strains were not suitable for industry-scale vaccine production due to their low efficient growth in ECE.

An acceptable approach is to change HP H5N1 phenotype to LP H5N1 by removal of the polybasic amino acids (RRRKKR) located between HA1 and HA2 of HA molecules (Treanor *et al*., 2006). HG seed viruses were generated by using plasmid-based reverse genetics (Hoffmann *et al*., 2000; Nicolson *et al*., 2005). Indeed, six internal protein genes (PA, PB1, PB2, M, NP and NS) derived from high growth virus A/Puerto Rico/8/34 (H1N1) and two genes (modified HA and unmodified NA) of the circulating HP H5N1virus strains were cloned into plasmids. They are co-transfected into mammalian cell lines such as 293T (Liu *et al*., 2003; Subbarao *et al*., 2003), MDCK cells (Medema *et al*., 2006) and Africa green monkey Vero cells (Nicolson *et al*., 2005) to rescue seed viruses. Recently, a novel approach use Vero cells as substrates to propagate wild-type HP H5N1 virus (Ehrlich *et al*., 2008) without any gene modification.

There are two types of vaccines developed to cope with the next influenza pandemic: (1) pandemic vaccines that are used only in pandemic. Pandemic vaccines must contain viruses matched to causative strain. (2) prepandemic vaccines that contain an influenza virus which has the potential to cause a pandemic. These vaccines can be used to prime populations during both the interpandemic phase (phase 1 and 2) and the pandemic alert period (phase 3, 4 and 5) (O'Neill and Donis, 2009; Palache and Krause, 2009). In fact, it is not possible to determine or predict which virus strains will cause a next pandemic. The European Medicines Evaluation Agency (EMEA) announced a new procedure that allows and facilitates influenza vaccine manufacturers to gain an authorization for evaluation of mock-up/pandemic vaccines before a pandemic has occurred (EMEA, 2006). Mock-up/pandemic vaccines contain influenza virus strains that mimic the future pandemic influenza viruses but do not circulate in humans. All data referring to preclinical, clinical studies, manufacturing method, quality, quantity and compositions of mock-up vaccines/pandemic vaccines are required to receive approvals. Using mock-up/pandemic vaccines, the manufacturers are able to predict how human population will have immune responses with future pandemic viruses (EMEA, 2006; Palache and Krause, 2009). When a pandemic occurs, variation with new virus strain in pandemic vaccine will be re-submitted. It will be processed very quickly (~3 days). Currently, HP H5N1 influenza viruses which belong to clade 1 including A/Vietnam/1194/2004(H5N1) and A/Vietnam/1203/2004 (H5N1) have been used in mock-up/pandemic vaccines. Since 2005, 12

pandemic vaccines have licensed and received approvals (http://www.who.int/vaccine_ research/diseases/influenza/flu_trials_tables/en/; Palache and Krause, 2009) and shown in table I-1.

Table I-1. Mock-up/pandemic vaccines. Wt VN/1203*: wild type A/Vietnam/1203/2004(H5N1); Inac VN/1194*: inactivated A/Vietnam/1194/2004(H5N1); GSK: GlaxoSmithKline Biologicals; EMEA: the European Medicines Evaluation Agency; CSL: the Commonwealth Serum Laboratories.

Producer (Trade mark)	**Virus strain**	**Adjuvant and dose**	**Type of vaccine substrate**	**Reference Regulatory status**
Baxter (Celvapan®)	Wt VN/1203*	No adjuvant 2 doses of 7.5 µg	Whole virus Vero cells	(Ehrlich *et al.*, 2008) Europe approval, 2009
Sanofi Pasteur	Inac VN/1203*	No adjuvant 2 doses of 90 µg	Split Eggs	(Treanor *et al.*, 2006) USA approval, 2007
Sanofi Pasteur (Emerflu®)	Inac VN/1194*	$Al(OH)_3$ 2 doses of 30 µg	Split Eggs	(Bresson *et al.*, 2006) Australia approval, 2009
Biken (BK PIFA®)	Inac VN/1194*	$Al(OH)_3$ 2 doses of 15 µg	Whole virus Eggs	Japan approval, oct/07
Kaketsuken	Inac VN/1194*	$Al(OH)_3$ 2 doses of 15 µg	Whole virus Eggs	Japan approval
Kitasato Institute	Inac VN/1194*	$Al(OH)_3$ 2 doses of 15 µg	Whole virus Eggs	Japan approval, oct/07
Denka Seiken	Inac VN/1194*	$Al(OH)_3$ 2 doses of 15 µg	Whole virus Eggs	Japan approval
Sinovac (Panflu®)	Inac VN/1194*	$Al(OH)_3$ 2 doses of 10 µg	Whole virus Eggs	(Wu *et al.*, 2009) China approval, apr/08
GSK biologicals (Daronrix®)	Inac VN/1194*	$Al(OH)_3$ 2 doses of 15 µg	Whole virus Eggs	Europe approval, mar/08
GSK biologicals (Pandemix®)	Inac VN/1194*	AS03 2 doses of 3.75 µg	Split Eggs	(Schwarz *et al.*, 2009) Europe approval, may/08
CSL Limited (Panvax®)	Inac VN/1194*	$Al(OH)_3$ 2 doses of 30 µg	Split Eggs	(Nolan *et al.*, 2008) Australia approval, 5/08
Novartis V&D (Focetria®)	Inac VN/1194*	MF59 2 doses of 7.5 µg	Subunit Eggs	(Banzhoff *et al.*, 2009) Europe approval, may/07

Most of the attenuated vaccine viruses were grown in ECEs as the traditional substrate to produce licensed mock-up/pandemic vaccines except the vaccine produced in Vero cells using wild-type virus A/Vietnam/1203/2004(H5N1) (Ehrlich *et al.*, 2008). The mock up/pandemic vaccines have been prepared as inactivated whole, split and subunit vaccines. All vaccines were well tolerated without serious adverse events (Bresson *et al.*, 2006). Mild pain at the injection site and headache were commonly observed (Baras *et al.*, 2008; Ehrlich *et al.*, 2008; Treanor *et al.*, 2006). Clinical results obtained from these mock-up/pandemic vaccines showed that both non adjuvanted (Treanor *et al.*, 2006) and aluminium adjuvanted split

vaccine (Bresson *et al*., 2006) were poorly immunogenic. A two-dose regime containing 90 µg of HA from non adjuvanted H5N1 split vaccine was needed to achieve a HI titer of 40 or greater in 57% vaccinees and a neutralization antibody titer of 40 or higher in 53% of subjects (Treanor *et al*., 2006). Another split vaccine targeting an A/Vietnam/1194/2004/NIBRG 14(H5N1) strain developed by Sanofi Pasteur showed that the only group that received 30 µg of the aluminium adjuvanted split influenza vaccine could induce the highest immune response with 67% HI seroconversion rate after two shots (Bresson *et al*., 2006).

Whole inactivated virus vaccines were also conducted by several manufacturers (Table I-1). In general, these vaccines were more immunogenic than split vaccines. These vaccines were well tolerant. Two doses (HA content ranging from 7.5 µg/dose (Ehrlich *et al*., 2008) to 10 µg/dose (Wu *et al*., 2009) and 15 µg/dose (Table I-1) were necessary to elicit protective antibodies. Specially, Ehrlich and co-workers reported that the aluminium adjuvanted vaccines reduced immunogenicity of the vaccines (Ehrlich *et al*., 2008). The results showed that the formulations with non adjuvanted 7.5 and 15 µg dosages after second immunization conferred the highest rates of a virus-neutralization titer of 1:20 or greater in 76 and 70%, respectively. These data as well as results from aluminium adjuvanted split vaccines showed that aluminium adjuvant has very little effect on influenza immunogenic vaccine.

With current influenza vaccine production using substrates including ECE and Vero cells, the first lots of pandemic vaccines are possibly available between 3 months (cell-based vaccine production) and 6 months (egg-based vaccine production) after the virus is determined (Wright, 2008). In addition, based on computer models focusing on USA and UK, pandemic influenza viruses would spread nationwide only within 1 month and influenza outbreak could be reached 60-80 days in USA and 50-60 days in UK after the first case (Ferguson *et al*., 2006; Germann *et al*., 2006; Palache and Krause, 2009). Pandemic vaccines will be difficultly available in first wave outbreak of pandemic virus strains. Therefore, prepandemic vaccines will play a key role in mitigating an influenza pandemic (Palache and Krause, 2009). Although prepandemic vaccines do not contain a causative virus, they will provide population primed by prepandemic vaccines some memory immune responses with some cross-activity with the next pandemic viruses. Priming population will eventually be boosted by pandemic vaccines when a pandemic is declared by WHO (Palache and Krause, 2009; Rappuoli and Giudice, 2008). This option is very useful in term of using oil-in-water adjuvants that have been shown to induce cross-protection (Rappuoli and Giudice, 2008; Schwarz *et al*., 2009; Vogel *et al*., 2009).

Table I-2. Prepandemic vaccines. Wt VN/1203*: wild type A/Vietnam/1203/2004(H5N1); Inac VN/1194*: inactivated A/Vietnam/1194/2004(H5N1); GSK: GlaxoSmithKline Biologicals; EMEA: the European Medicines Evaluation Agency; CSL: the Commonwealth Serum Laboratories; MHLW: Ministry of Health, Labor and Welfare (Japan).

Producer (Trade mark)	**Virus strain**	**Adjuvant dose**	**Type of vaccine substrate**	**Reference Regulatory status**
Sinovac (Panflu®)	Inac VN/1194*	$Al(OH)_3$ 2 doses of 10 µg	Whole virus Eggs	(Wu *et al.*, 2009) China approval, apr/08
Biken (BK PIFA®)	Inac VN/1194*	$Al(OH)_3$ 2 doses of 15 µg	Whole virus Eggs	Japan approval, oct/07
Kitasato Institute	Inac VN/1194*	$Al(OH)_3$ 2 doses of 15 µg	Whole virus Eggs	Japan approval, oct/07
Omninvest (Fluval®)	Inac VN/1194*	$Al(OH)_3$ 2 doses of 6 µg	Whole virus Eggs	(Vajo *et al.*, 2007) Hungarian approval, 9/08
GSK biologicals (Prepandrix®)	Inac VN/1194*	AS03 2 doses of 3.75 µg	Split Eggs	(Schwarz *et al.*, 2009) European, Malaysia, HK approval, 5/08
Baxter (Celvapan®)	Wt VN/1203*	No adjuvant 2 doses of 7.5 µg	Whole virus Vero cells	Submission planned 2009
Denka Seiken	Inac VN/1194*	$Al(OH)_3$ 2 doses of 15 µg	Whole virus Eggs	Submitted to MHLW 01/2007
Kaketsuken	Inac VN/1194*	$Al(OH)_3$ 2 doses of 15 µg	Whole virus Eggs	Submitted to MHLW 04/2008
Novartis V&D (Focetria®)	Inac VN/1194*	MF59 2 doses of 7.5 µg	Subunit Eggs	Submission planned 2009

As a result of scientific activities, five out of nine prepandemic vaccines have received approvals and listed in table I-2 (www.who.int/immunization/sage/SAGE_H5N1_26Mayb.pdf; Palache and Krause, 2009). Currently, the H5N1 prepandemic vaccines are destined to public health national vaccine stockpiles at many countries such as USA (40 million doses, 90 µg/dose), Switzerland (8 million doses), Japan (20 million doses), Finland (5.2 million doses), WHO (140 million doses) and other countries with a total of around 230,3 million doses (for review see Palache and Krause, 2009).

I.1.3.2.2. Live-attenuated influenza vaccines

Live-attenuated influenza vaccines (LAIV) are also cold-adapted influenza vaccine (Fiore *et al.*, 2009). The viruses used to produce LAIV display two important phenotypes: (1) cold-adapted, temperature sensitive: they grow efficiently at 25 °C and 33 °C (ca phenotype) but not higher than 39 °C (ts phenotype) (Maassab, 1969); (2) attenuated (att phenotype): viruses can not replicate efficiently in lower respiratory tract, therefore they can not cause severe illness in humans (Maassab and Bryant, 1999) and experimental animals (Maassab *et*

al., 1982). These phenotypes are the results of virus serial passages at progressively lower temperature. The ca, ts and att phenotype harboring viruses are restricted to replicate in the respiratory tract, therefore it is unlikely that they pose systematic symptoms of influenza. To produce seasonal LAIV, ca, ts and att viruses from either influenza type A or influenza type B are selected to serve as master donor viruses (MDV) for vaccine reassortants (Fiore *et al*., 2009) such as A/Ann Arbor/6/60(H2N2) and influenza B/Ann Arbor/6/60 available in the United States or A/Leningrad/137/17/57ca (H2N2) (Alexandrova and Smorodintsev, 1965), A/Leningrad/137/47/57ca (H2N2) (Garmashova *et al*., 1984) and B/USSSR/60/69 available in Russia (Kendal, 1997; for review see Chen and Subbarao, 2009). Cold-adapted, temperature sensitive and attenuated reassortant viruses are generated by classical genetic reassortment. In recent years, reverse genetics technology has been used to substitute the former procedures (Fiore *et al*., 2009). The reassortant viruses contain HA and NA genes from currently circulating viruses and 6 internal genes from the cold adapted MDV (Chen and Subbarao, 2009; Fiore *et al*., 2009). Unlike whole inactivated viruses, LAIVs were administrated intranasally. Currently, trivalent LAIVs are licensed to protect human population from seasonal influenza. Two LAIV vaccines have been licensed in Russia and one, FluMist®, has been licensed in the USA since 2003 (Ambrose *et al*., 2008; Hickling and D'Hondt, 2006).

LAIV was also developed to fight against HP H5N1 influenza viruses. Several preclinical studies were performed to evaluate live attenuated H5N1 candidates. Vaccine viruses containing modified HA (remove cleavage site) and unmodified NA from A/HK/156/1997(H5N1), A/HK/483/1997(H5N1) (Li *et al*., 1999); A/VN/1203/2004(H5N1), A/HK/213/2003(H5N1), A/HK/486/1997(H5N1) (Suguitan *et al*., 2006) and 6 genes from master donor virus A/Ann Arbor/6/60(H2N2) were generated by using reverse genetics technique. These recombinant viruses replicated to high titer in ECE and still remained attenuated phenotype in ferrets (Li *et al*., 1999; Suguitan *et al*., 2006) or in mice (Suguitan *et al*., 2006). More importantly, mice vaccinated with single dose (dose of 106 TCID50) of these vaccines were fully protected from wild type homologous and heterologous H5N1 viruses at 50, 500, 5000 LD50 (Suguitan *et al*., 2006), although neutralizing serum antibodies were undetectable. Wild type viruses were dectected in the respiratory tract after single dose with lower titer than in mock group, but not after two doses (Suguitan *et al*., 2006).

In 3/2009, a H5N2 LAIV pandemic vaccine candidate was licensed in Russia (Trade mark: Ultraarivac). This high growth 7:1 vaccine reassortant was generated by reverse genetics using the hemagglutinin (HA) gene from non-pathogenic A/Duck/Potsdam/1402-6/86 (H5N2)

virus and the seven genes from the cold-adapted (ca) attenuated A/Leningrad/134/17/57 (H2H2) strain (Desheva *et al*., 2006). Two dose of H5N2 LAIV at 108.3 TCID50 elicited a four-fold rise in HI titers in 74% of the subjects (HI to A/Vietnam/1194/2004(H5N1), clade 1) and 71.9% of the subjects (HI to A/Indonesia/5/2005(H5N1), clade 2.1) (Zverev *et al*., 2009). Another trial with healthy adults was conducted by Karron and co-workers showed that live attenuated H5N1 vaccines were very restricted in replication and very attenuated in comparison to seasonal LAIV. These resulted in undetected HI and neutralizing antibodies (Karron *et al*., 2009). Although LAIVs have been licensed for seasonal vaccines and intended to use for pandemic vaccination, LAIVs containing HA and NA from HP H5N1 viruses are not recommended as prepandemic vaccines. They are considered to use only in pandemic stage to avoid any possibility of reassortment (Hickling and D'Hondt, 2006).

I.1.4. Adjuvants for influenza vaccines

The function of adjuvants is to improve an immunogenicity of vaccines with different ways such as increasing interaction time between antigens and the immune system, facilitating the delivery of antigens to antigen presenting cells (Atmar and Keitel, 2009). Adjuvants that have been used for influenza vaccines are mineral salts, emulsion, particulates and others (Atmar and Keitel, 2009).

Aluminium salts are the only adjuvant which is currently licensed in the Unite States for human vaccines. Using these adjuvants for influenza vaccines has been evaluated since 1976. The adjuvant aluminium effect has been controversial with contrary results. Some studies showed that modest improvement in immune responses was observed (Nicholson *et al*., 1979; Pressler *et al*., 1982), but other studies reported that the difference of antibody responses between aluminium adsorbed and unadsorbed vaccines was not significant (Davenport *et al*., 1968). The effect of aluminium adjuvant with H5N1 influenza vaccines caused slight increase in immune response. It was observed only in groups that received a high dose of subvirion H5N1 influenza vaccine (Bresson *et al*., 2006). Ehrlich and co-workers demonstrated that aluminium adjuvant even reduced immunogenicity of the whole virus vaccines in adjuvanted vaccines (Ehrlich *et al*., 2008).

Emulsion based-adjuvants are another adjuvant system that can be used for influenza vaccines. Emulsion is generated by mixing water-solubilized and oil-solubilized components with emulsifiers which have a role in stabilizing resulting emulsions (Atmar and Keitel, 2009). Freund (Freund and Opie, 1938), Montanide ISA51 and 65 are three popular water-in-oil adjuvants (Atmar and Keitel, 2009). Freund and Opie developed a water-in-oil adjuvant

including killed mycobacteria and Arlacel A employed as emulsifiers. This was called complete Freund's adjuvant (CFA). When killed mycobacteria are removed, incomplete Freund's adjuvant (IFA) is formed and adjuvant activity is still remained. In the past, a seasonal influenza vaccine adjuvanted with IFA was licensed in the UK. This IFA adjuvanted influenza vaccine could induce higher immune responses and approximately 1 million doses of this vaccine were injected in the UK during two years (Perkins *et al*., 1972, for review see Vogel *et al*., 2009). Currently, these adjuvants are commonly used for preclinical research in animals because they have been too toxic for human use (Edelman, 1980). Toxicity of water-in-oil is reduced when the amount of oil is reduced from 50 to 4-5%. Mineral oil and Arlacel surfactant (generally used in water-in-oil) is replaced by squalene oil and Tween 80 or Span 85, respectively. These changes result in generating of new oil-in-water adjuvant systems. Currently, MF59 (developed by Novartis V&D, Podda, 2001), AS03 (developed by GlaxoSmithKline[GSK], Leroux-Roels *et al*., 2007) and AF03 (developed by Sanofi Pasteur, Levie *et al*., 2008) are three popular oil-in-water adjuvants that are used as adjuvants for influenza vaccines (Vogel *et al*., 2009). The recent reports showed that the formulation of mock-up/pandemic (Table I-1), prepandemic influenza vaccines (Table I-2) and seasonal vaccines with oil-in-water adjuvants not only decreases the significant HA dose (as low as 1.9 µg (Levie *et al*., 2008), 3.8 µg (Leroux-Roels *et al*., 2007) or 7.5 µg (Banzhoff *et al*., 2009)) but also improves immune responses.

The other adjuvant system evaluated as new potential adjuvant for influenza vaccines is the ISCOM technology-based Matrix M™ adjuvant (Lövgren Bengtsson *et al*., 2011). The immune stimulating complexes (ISCOMs) form the first generation adjuvant which is a mixture of antigen, cholesterol, saponins and phospholipid to form stably spherical particles of approximately 40 nm in diameter (Lövgren Bengtsson *et al*., 2011; Sun *et al*., 2009). The secondary generation adjuvant, ISCOMATRIX, is also a particulate adjuvant comprising cholesterol, saponin and phospholipid but without antigen (Sjolander *et al*., 1998; Sun *et al*., 2009). ISCOMs and ISCOMATRIX vaccines induced strongly both humoral and cellular immune responses (Drane *et al*., 2007; Sjolander *et al*., 2001) as well as improving of antigen uptake by antigen present cells (APCs) (Atmar and Keitel, 2009). These adjuvants are limited use for human trials due to the toxicity of saponin. The major source saponin used in ISCOMs and ISCOMATRIX is Quil A extracted from *Quillaja saponaria* Molina. Quil A contains probably more than 100 closely related saponins (Sun *et al*., 2009) suitable to use for veterinary vaccination. For human application, purified and well-defined fractions of saponin are used. A combination between the purified saponin fraction A and fraction C with specific

ratios was referred as the third generation immune stimulating complex Matrix M™ (Lövgren Bengtsson *et al*., 2011). The Matrix M™ was evaluated as the adjuvant allowing dose reduction to a level of 1.5 µg HA (Cox *et al*., 2011).

I.2. Molecular pharming in plants

I.2.1. Plant as platform for production of pharmaceutical proteins

Worldwide outbreaks of infectious diseases such as influenza virus, HIV (Human immunodeficiency virus), SARS (severe acute respiratory syndrome) and rapid growth of human population have led to increased demands of vaccines, antibodies, human hormones and enzymes (for review see Ma *et al*., 2005; Twyman, 2003). Most of these therapeutic products have been produced in *E. coli* and mammalian cells which have referred to be technically complex, slow and expensive (for review see Twyman *et al*., 2005). Hence, the potential collapse of the therapeutic proteins and high cost products produced by established expression system from 1980s have provided a strong rationale for using alternative expression systems such as plants (for review see Fischer and Emans, 2000; Ma *et al*., 2005; Twyman, 2003), plant cell cultures (for review see Hellwig *et al*., 2004), filamentous fungi and yeast cells (Gerngross, 2004) in pharmaceutical protein production.

In these alternative expression systems, plants have emerged as a potential means to provide therapeutic proteins. Early recombinant proteins successfully expressed in transgenic plants were human growth hormone (Barta *et al*., 1986), interferon (De Zoeten *et al*., 1989) and human serum albumin (Sijmons *et al*., 1990). The main advantages of this expression system are: (1) recombinant protein production at low enough cost due to using well established existing infrastructures (Giddings, 2001; Schillberg *et al*., 2003); (2) the easy up-scaling just by enlarging greenhouse space or land for transgenic plants as required (for review see Yusibov and Rabindran, 2008); (3) avoidance of contamination with human pathogens (Fischer and Emans, 2000); (4) eukaryotic protein modification machinery allowing subcellular targeting, proper folding and post-translational modifications in plant cells (Fischer and Emans, 2000); (5) high-level expression, easy and long-term storage of recombinant proteins in seeds (Boothe *et al*., 2010; Stoger *et al*., 2005).

Recombinant proteins could be produced by either stable genomic integration or transient expression (Mason and Arntzen, 1995). Transient expression is a flexible method to express proteins in short time without chromosomal positional effects (Fischer *et al*., 1999). Leafy crops such as tobacco, alfalfa and lettuce are suitable for this expression system.

DNA/vectors were introduced into plant cells by the following ways: (1) delivery of ‘‘naked’’ DNA by particle bombardment, (2) infection with modified viral vectors (Gleba *et al*., 2007) and (3) infiltration of plant tissue with recombinant Agrobacterium (agroinfiltration) (Fischer and Emans, 2000; Negrouk *et al*., 2005). Transient expression systems could provide enough recombinant proteins for pre-clinical trials (Fischer and Emans, 2000) and are suitable to produce influenza pandemic vaccines that are required to be produced in short time for use of huge doses in case of an influenza pandemic outbreak (D'Aoust *et al*., 2010). In contrast to transient expression systems, stable transformation is defined by the integration of the target genes into the host plant genome. Agrobacterium mediated gene transfer is the most widely used technique for dicot transformation such as tobacco (Fischer and Emans, 2000) and pea (Prasad *et al*., 2004). In addition, bombardment-based technology also has been used to introduce transgenes into host plant genome (Christou, 1997). Normally, stable transformation of plants can take 3-9 months before plants are available for testing expression of recombinant proteins. The transformation process depends on the plant species to be transformed.

In 1989, Hiatt and co-workers reported that complete antibodies could be produced in transgenic tobacco (Hiatt *et al*., 1989). Since then, a number of antibodies (complete antibodies (Ma *et al*., 1994), single chain Fv fragments (Artsaenko *et al*., 1998; Fiedler *et al*., 1997; Fischer *et al*., 1999; Joensuu *et al*., 2009; Owen *et al*., 1992) and Fab fragments (De Wilde *et al*., 1996; Peeters *et al*., 2001) have been produced in a range of different plants (for review see Schillberg *et al*., 2003) including tobacco plants (Floss *et al*., 2008; Villani *et al*., 2009), maize (Rademacher *et al*., 2008) and pea (Prasad *et al*., 2004). Although more than 100 therapeutic proteins have been expressed in transgenic plants including antibodies, antigens, cytokines, growth hormones, recombinant enzymes over the past 20 years (for review see Twyman *et al*., 2005), only few antibodies are currently investigated in clinical trials. The first case is the antibody with CaroRx™ trademark against Streptococcus mutans causing tooth decay developed by Planet Biotechnology Inc. (Ma *et al*., 1998). Other examples are various single chain Fv antibody fragments used to treat Non-Hodgkin's lymphoma (McCormick *et al*., 2008) and the monoclonal 2G12 antibody neutralizing HIV (Fox, 2011). Besides antibodies, several recombinant proteins from plants are approaching to commercialization including Gastric lipase (Meristem Therapeutic) for cystic fibrosis pancreatitis treatment, Human intrinsic factor for vitamin B12 deficiency treatment, Lactoferrin (Meristem Therapeutic) for gastrointestinal infection treatment (Ma *et al*., 2005) and several vaccines (section I.2.2).

I.2.2. Plant-derived vaccines

Vaccination continues to be the most economically feasible and the most effective approach to protect animals and humans from many infectious diseases. Currently, the majority of licensed bacterial and viral vaccines are either live attenuated or killed causative agents. However, the development of modern molecular biological tools, genetic engineering and genomics, combined with a better understanding of not only which antigens are important for inducing protection, but also an appreciation of host defenses that must be stimulated, has opened a novel strategy to develop safer and more effective vaccines: subunit vaccines (Rogan and Babiuk, 2005). Numerous subunit vaccines have been successfully produced in various expression systems including bacterial, yeast, insect cell, viral, mammalian cell and plant-based expression systems (for review see Fischer and Emans, 2000; Rogan and Babiuk, 2005).

The first proof of concept for expression of an antigen in plants was established in 1990 when the surface protein antigen A (SpaA) of *Streptococus mutans* causing tooth decay was expressed in transgenic tobacco plants (Curtiss and Cardineau, 1990). Mice were fed with a mixture of the transgenic tobacco tissue expressing SpaA and their diet. Mucosal immune responses were induced by SpaA from transgenic plants. These antibodies were biologically active and reacted with intact *S. mutant*. The successful research was patented in 1990 (Curtiss and Cardineau, 1990). Subsequently, Mason and co-workers successfully expressed the Hepatitis B virus surface antigen (HBsAg) and they introduced the concept of "edible vaccines" (Mason *et al.*, 1992). Since then, more than 67 different antigens of 24 different pathogens have been expressed in different plant species (Floss *et al*., 2007). These include the expression of a rabies virus glycoprotein in tomatoes (McGarvey *et al.*, 1995); a cholera antigen in potatoes, tomatoes (Arakawa *et al.*, 1998; Arakawa *et al.*, 1997; Jani *et al.*, 2002; Jani *et al.*, 2004); Norwalk virus capsid protein in tobacco and potato (Mason *et al.*, 1996); ESAT-6 of mycobacterium tuberculosis in *A. thaliana* (Rigano *et al*., 2004) and *N. tabacum* (Floss *et al.*, 2010a; Zelada *et al.*, 2006) etc..

Using plants as an alternative substrate to produce hemagglutinin-based subunit vaccines has become the focus of many researchers worldwide in recent years. Recent reports showed that the plant-derived influenza vaccines were safe and effective in preclinical tests and easy to scale-up (D'Aoust *et al*., 2008; D'Aoust *et al*., 2010; Chichester *et al.*, 2009; Yusibov and Rabindran, 2008). The early attempt to express a main surface glycoprotein hemagglutinin was reported by Chandler. Influenza hemagglutinin subtype 3 (HA3) was expressed permanently in tobacco plants with low expression level. Results of downstream

processing were not included in this thesis (Chandler, 2007). Subsequently, the stem (amino acids 17-58 and 293-535) and the globular (amino acid 59-292) domains as well as N1 of hemagglutinin subtype 5 from A/Vietnam/1194(H5N1) and A/Wyoming/3/03(H3N2) were produced in fusion with the carrier protein lichenase (LicKM). Recombinant proteins were purified by 6x his tag-based chromatography and could elicit high HI titers, virus neutralizing antibody titers. Ferrets that were vaccinated with these proteins were protected from homologous virus (Mett *et al.*, 2008; Musiychuk *et al.*, 2007).

In subsequent studies, hemagglutinin ectodomains from different strains (A/Wyoming/3/ 03(H3N2), A/Indonesia/05/2005(H5N1), A/Bar-headed Goose/Qinghai/1A/05 (H5N1) and A/Anhui/1/05(H5N1)) were also expressed in *N. benthamiana*. These candidate vaccines could protect animals from homologous viruses and provide some cross-activity conducted by HI and neutralizing titers (Shoji *et al.*, 2009a; Shoji *et al.*, 2008; Shoji *et al.*, 2009b). In these studies, vaccines were formulated with Quil A adjuvant.

Another interesting approach for influenza vaccine production in plants uses hemagglutinin-based-virus-like particles. D'Aoust and co-workers reported that only full hemagglutinin from A/Indonesia/05/2005(H5N1) could produce virus-like particles with typical size and morphology of influenza viruses. This candidate vaccine provided protection against challenge with 1 LD_{50} of heterologous virus A/Vietnam/1194(H5N1) at dose as low as 0.5 µg (D'Aoust *et al.*, 2008). A recent report by Kalthoff and co-workers showed that the membrane-bound hemagglutinin was produced in *N. benthamiana* using the magnICON (Icon Genetics, Halle, Germany) provector system. This candidate vaccine protected partially chickens (approximately of 90%) that were vaccinated with Freund and BAY98-7089-adjuvanted vaccine from lethal virus and protected fully vaccinated chickens from lethal virus using Polygen adjuvant (Kalthoff *et al.*, 2010). Although many recombinant proteins were successfully produced in various plant species, only four vaccines (for review see Ma *et al.*, 2005) and a plant-derived influenza VLP vaccine (Landry *et al.*, 2010) have entered clinical trials. A veterinary vaccine for Newcastle disease prevention has been approved in recent years (Walsh, 2006).

Because it is impossible to predict which influenza virus strains will cause a next pandemic outbreak (section I.1.3), it is very important to have an expression system which is able to provide vaccine volume meeting increasing demands in short time (pandemic case). Transient expression using *N. benthamiana* is a promising system in this context.

I.3. Elastin-like polypeptides

Elastin, the extracellular matrix protein, plays an important role in the structure and is responsible for flexibility and elasticity in several connective organs (the lung, skin, blood vessels and ligaments) (Keeley *et al*., 2002). The elastin is insoluble, however, its precursor, tropoelastin with a molecular weight approximately 72 kDa, is highly soluble (Vrhovski and Weiss, 1998). The tropoelastin contains two alternately main domains: (1) the hydrophilic domain which is a K, A rich domain that is important in crosslinking formation through the activity of the copper-requiring enzyme lysyl oxidase; (2) the hydrophobic domain which is rich in non-polar amino acids, especially V, P, G and A. They often occur in repeats of four to six peptides such as VPGG, VPGVG and APGVGV providing the elasticity of the protein. The number of the repeat units is different between species (Gray *et al.*, 1973; Vrhovski and Weiss, 1998; for review see Floss *et al.*, 2010b).

Because of insolubility of native elastin, many of these repetitive patterns were synthesized to elucidate their physical and functional properties (McPherson *et al.*, 1992; Urry, 1992). Recently, (VPGXG)n, termed as "elastin-like polypeptides" (ELPs), has been attracted much attention due to its potential uses for drug deliver (Meyer *et al.*, 2001), protein purification (Meyer and Chilkoti, 1999; for review see Floss *et al.*, 2010b) and tissue engineering (Hrabchak *et al.*, 2010; McHale *et al.*, 2005; Ong *et al.*, 2006; Nettles *et al.*, 2010; for review see MacEwan and Chilkoti, 2010). ELPs consist of repeats of the pentapeptide sequence VPGXG, where the guest residue X is any amino acid residue except P (Meyer and Chilkoti, 1999). This sequence is derived from the characteristic repeat motif, VPGVG, found in all native mammalian elastin proteins (Floss *et al.*, 2010b; Gray *et al.*, 1973; Vrhovski and Weiss, 1998; Trabbic-Carlson *et al.*, 2004). ELPs undergo an inverse phase transition. They are highly soluble in water below the inverse transition temperature (T_t) and exist as monomers. When the temperature is raised above T_t, desolvation and aggregation of the polypeptides occur and they become insoluble (Urry, 1988; Urry, 1992; Urry, 1997). The T_t is dependent on several factors such as the properties of accompanying fusion proteins, ELP chain length, ELP concentration (Meyer and Chilkoti, 2004), salt concentration (Luan *et al.*, 1991) and pH (Li and Daggett, 2003; MacKay *et al.*, 2010).

Because the T_t of ELPs is reversible, Meyer and Chilkoti exploited the phase transition induced aggregation of ELP to develop a new protein purification method termed "inverse transition cycling" (ITC) for enrichment of ELP fusion proteins (Meyer and Chilkoti, 1999). When the transition of ELP fused proteins are triggered by raising temperature up to 35-45 °C

and adding NaCl (≤ 2M), the fusion protein aggregates were separated from contaminant proteins by centrifugation at thermal condition, 35-45 °C. The pelleted ELP fusion proteins can be resolubilized by using cold, low-ionic-strength buffer. The ELP based enrichment protein method was applied successfully to purify several ELP fused proteins expressed in *E. coli* (Christensen *et al.*, 2009; Shimazu *et al.*, 2003; Trabbic-Carlson *et al.*, 2004). The temperature dependent purification method was also applied successfully to purify ELP fusion proteins in plants (referred to as ELPylation (Floss *et al.*, 2010b)) either from leaves (Conley *et al.*, 2009a; Conrad *et al.*, 2011; Floss *et al.*, 2010a; Floss *et al.*, 2008; Scheller *et al.*, 2004) or seed materials (Floss *et al.*, 2009; Schallau, 2008). The ELPylated proteins expressed in *E. coli* were also separated selectively by using membranes with high recovery rate, firstly developed by Ge and co-workers (Ge *et al.*, 2006). It was then applied to purified recombinant proteins expressed in *E. coli* at low expression levels (Christensen *et al.*, 2007).

In plant, the ELP fusion strategy not only facilitates an easy way for protein purification by ITC but also results in enhancing expression level of recombinant proteins. Scheller and co-workers generated the fusion protein of scFv and 100 repeats of the ELP motif that targets in seeds. It was shown that the accumulation of this fusion protein dramatically increased up to a level 40-fold greater than that of unfused scFv approximately 25% TSP (Scheller *et al.*, 2006). Floss and co-workers used the same approach and proved that the ELP fusion strategy could enhance the expression level of HIV neutralizing antibodies expressing in seeds (Floss *et al.*, 2009). ELPs fused to C-terminal recombinant proteins also could enhance the accumulation of many proteins expressed in leaves (Conley *et al.*, 2009a; Conrad *et al.*, 2011; Floss *et al.*, 2009; Patel *et al.*, 2007). Interestingly, the ELP fusion approach can remain functionalities of ELP fused proteins (Conrad *et al.*, 2011; Floss *et al.*, 2009; Ge *et al.*, 2006).

After ELP fused proteins are purified by ITC, ELP tags can be removed to obtain pure target proteins. Two strategies using either self-cleaving intein (Banki and Wood, 2005; Banki *et al.*, 2005) or ELP fused proteases (Lan *et al.*, 2011) without the need for affinity chromatography were applied efficiently for this purpose. The use of self-cleaving intein is a promising method which is able to produce huge amounts of high valuable proteins because of its ease to scale up and reducing cost for enrichment of intact recombinant proteins.

I.4. The aims of the thesis

Influenza hemagglutinin and neuraminidase are two target surface glycoproteins to develop influenza subunit vaccines and split vaccines, because they are able to elicit neutralizing antibodies that could prevent influenza virus entry and replication. In this thesis, hemagglutinin and neuraminidase (monomer and oligomer) from the HPAI A/Hatay/2004/(H5N1) virus strain are optimally expressed in terms of their functionalities and protein yields by fusing with elastin-like polypeptides. Recombinant proteins are produced in both stable transgenic plants and transiently transformed *N. benthamiana* plants. The effect of ELP-fusion technology on the accumulation of recombinant influenza hemagglutinin and neuraminidase in plants is evaluated both in seeds and leaves of transgenic tobacco plants as well as in *N. benthamiana*.

The ELP-fusion technology also provides a simple and inexpensive means for purification of ELP fused proteins by using inverse transition cycling (ITC). It is necessary to optimize a specific purification procedure for each ELP fused protein to enrich it efficiently. To this end, ELP fused hemagglutinin and neuraminidase proteins are purified from leaf materials by using centrifugation-based ITC (cITC) and by using the improved protocol, membrane-based ITC (mITC).

Purified influenza hemagglutinin and neuraminidase are characterized biochemically and biofunctionally. Hemagglutinins fused with 100xELP and without fusion are characterized functionally by hemagglutination assay which is based on the ability to agglutinate chicken erythrocytes of hemagglutinin. While the enzymatic activity of ELP fused neuraminidases is determined using 2'-(4-methylumbelliferyl)-α-D-N-acetylneuraminic acid as a substrate.

Evaluation of monomer and oligomer of hemagglutinins as well as their ELP fused hemagglutinins is conducted in chicken and mouse vaccination with two different adjuvants including Freund's adjuvant and ISCOM-based Matrix M^{TM} adjuvant (abISCO). Hemagglutination inhibition tests and ELISA are also carried out to measure neutralizing antibodies and total IgG antibodies, respectively.

II. Materials and Methods

II.1. Materials

II.1.1. Bacterial strains

Escherichia coli: XL1- Blue MR{ *recA1, endA1 gyrA96 thi-1, hsdR17, supE44 relA1 lac [F´proAB lac1qZDM15 Tn10 (Tetr)]*} was used as host cells for cloning steps. *Agrobacterium tumefaciens*: C58C1 (pGV2260 in C58C1; (Deblaere *et al*., 1985)) was used for Agrobacterium-mediated gene transfer and transient assay. *Agrobacterium tumefaciens* C58C1 bearing FUS 3 transcription factor vector was used to be transiently co-expressed with Agrobacteria containing binary vectors for expression of recombinant proteins under control of the seed specific USP promoter (Reidt *et al*., 2000).

II.1.2. Vectors and genes

Plasmid vectors: 100xELP sequence in pRTRA vectors was synthesized and described in detail by Scheller and co-workers (Scheller *et al*., 2004). pRTRA 35S-TBAG-100xELP, pRTRA 35S-TBAG vectors containing either 100xELP or no ELP, respectively were designed by Floss and co-workers (Floss *et al*., 2010a). pRTRA USP-scFv anti oxa-100xELP and pRTRA USP-scFv anti oxa were constructed by Scheller and co-workers (Scheller *et al*., 2006). These and other vectors are listed in table II-1.

Table II-1. Basic plasmids. TBAG: mycobacterial antigen TBAG coding gene; HA0: full hemagglutinin; NA: neuraminidase; USP: the seed specific USP promoter; 35S: the CaMV 35S promoter; 100xELP: 100 repeats of elastin-like polypeptides; Amp^r: ampicillin resistance; Kan^r: kanamycin resistance; scFv anti oxa: single chain variable fragment anti oxazolone; M1: influenza matrix protein 1.

Plasmid	Antibiotics	Origin
pRTRA 35S-TBAG-100xELP	Amp^r	(Floss *et al.*, 2010a)
pRTRA 35S-TBAG	Amp^r	(Floss *et al.*, 2010a)
pRTRA USP-scFv anti oxa-100xELP	Amp^r	(Scheller *et al.*, 2006)
pRTRA USP-scFv anti oxa	Amp^r	(Scheller *et al.*, 2006)
pPCRScript-opHA0	Amp^r	Synthesized by Geneart GmbH
pCR-NA	Amp^r	IBT, Vietnam; Genbank number AJ867075
pMA-pLI-pII	Amp^r	Synthesized by Geneart GmbH
pRTRA 35S- scFv anti M1	Amp^r	Unpublished
pCB301-Kan	Kan^r	Based on pCB301 (Xiang *et al.*, 1999)

Neuraminidase (N1) cDNA of A/Hatay/2004/(H5N1) strain was isolated from chicken at Hatay province, Vietnam in 2004, cloned into pCR2.1 vector, designated as pCR-NA and kindly provided by Molecular Microbiology, Institute of Biotechnology (IBT), Hanoi, Vietnam. Hemagglutinin (H5) sequence of A/Hatay/2004/(H5N1) strain and GCN4 trimerization domain (isoleucine zipper, pII)- GCN4 tetramerization domain (pLI) sequences were tobacco codon optimized, synthesized by Geneart GmBh and designated as pPCRScript-opHA0, pMA-pLI-pII, respectively (Table II-1). Amino acid sequences of H5 and N1 from the A/Hatay/2004/(H5N1) strain are available in the National Center for Biotechnology Information with accession number Q5QQ29 (HA) and Q5QQ28 (NA), respectively.

II.1.3. Plant materials

Nicotiana tabacum cv. Samsun NN (SNN) and *Nicotiana benthamiana*

II.1.4. Oligonucleotide primers

II.1.4.1. Oligonucleotide primers for PCR amplification

Table II-2. Primers for PCR. Sequences were written in 5' to 3' direction. F: forward primer; R: reverse primer; NA: neuraminidase; HA: hemagglutinin; underlines indicate recognition sequence of restriction enzymes; pII and pLI: GCN4-pII trimerization and GCN4-pLI tetramerization motives.

Name of primer	Sequence 5'-3'
ELP-HIS-c-myc-Esp3I-R	AGCGTCTCGGATCTATGGTGATGGTGGTGATGGGATCCCG
His-c-myc-Esp3I-R	AGCGTCTCGGGCCAAATGGTGATGGTGGTGATGCGCGGCCGC
HIS-c-myc-Esp3I-F	AGCGTCTCGGATCCGAGGTGCAGCTGTTGGA
NA-ELP-Esp3I-F	AGCGTCTCGGATCCATTCACACAGGGAATCAACACCAAG
NA-ELP-Esp3I-R	AGCGTCTCGGATCCCTTGTCAATGGTGAATGGCAA
NA-NotI-R	AGGCGGCCGCCTTGTCAATGGTGAATGGCAACTC
NA-pLI-ELP-Esp3I-F	AGCGTCTCGGATCCAGGATGAAGCAAATTGAAGATA
NA-pLI-ELP-Esp3I-R	AGCGTCTCGGATCCACCCTGGAAGTAAAGGTTCTCCTTG
NA-pLI-SalI-F	AAGAGTCGACATTCACACAGGGAATCAACAC
HA-Bam-F	AGGGATCCGATCAGATCTGCATTGGATACCA
HApII-pspOMI-R	TCTTGGGCCCCTCCAACTTAACACCAGAAATCT
HApII-ELP-Bam-R	GGTGATGGGATCCACCCTGGAAGTAAAGGTTCTCT
HA-Bam-R	AGGGATCCAATGCAAATTCTGCATTGTAACG
HA-NotI-R	AGGCGGCCGCAATGCAAATTCTGCATTGTAACG
HA0-NotI-R	TCTGCGGCCGCAATGCAGATCCTGCACTGAAG
opH2-BamR	AGGGATCCCTCCAACTTAACACCAGAAAT
opHA2-ELP-Bam-F	AGGGATCCCATCACTCTAACGAGCAAGGA
opHA1-Bam-R	AGGGATCCCCTTCTTTCTCTCTGTGGAGAGT
opHA1-NotI-R	AGGCGGCCGCCCTTCTTTCTCTCTGTGGAGAGT
VLP-HA-TAG-NotI-R	TCTGCGGCCGCCTAAATGCAGATCCTGCACTGAAG

II.1.4.2. Oligonucleotide primers for DNA sequencing

Table II-3. Primers for sequencing. Sequences were written in 5' to 3' direction, 35S: primer is annealed with the CaMV 35S promoter, USP: primer is annealed with the USP promoter, NA: neuraminidase; HA: hemagglutinin.

Name of primer	Sequence 5'-3'
35S-SQF	CACTGACGTAAGGGATGACGC
USP-SQF	TGACATCCACTTGGAGGATGC
35STerm	CTGGGAACTACTCACACA
HA-SQF1	TCTTTTGGTACTGTGGGGGATTC
HA-SQF2	ATAGCAATGAGCAGGGGAGTG
NA-SQF1	ATGGGGCTGTGGCTGTATTG

II.1.5. Media

II.1.5.1. Media for plant tissue culture and transient assay

MS medium (**M**urashige and **S**koog)

4.49 g/L Murashige and Skoog medium basal salt mixture including vitamins, microelements, 30 g/L sucrose, 0.9% agar (Difco, Detroit, USA) for solid medium, pH 5.8

Media were sterilized by autoclaving.

Infiltration buffer for transient assay

10 mM 2-(N-morpholino) ethanesulphonic acid MES, 10 mM MgSO4, pH 5.6

Buffer was prepared freshly.

II.1.5.2. Media for bacterial culture

LB medium (**L**uria-**B**ertani)

5 g/L Yeast extract, 10 g/L Tryptone, 10 g/L NaCl, 9 g/L Agar for solid medium, pH 7.0

SOC medium

5 g/L Yeast extract, 20 g/L Tryptone, 0.5 g/L NaCl, 0.186 g/L KCl, 20 mM glucose, pH 7.0

YEB medium

5 g/L Beef extract, 1 g/L Yeast extract, 5 g/L Peptone, 5 g/L Sucrose, 2 mM $MgSO_4$, pH 7.0. All media were sterilized by autoclaving.

II.1.6. Buffers

4x Marvel buffer pH 7.8 (4x TBS buffer pH 7.8)

80 mM Tris-HCl, 720 mM NaCl

Transfer buffer

10% methanol (v/v), 24 mM Tris, 194 mM Glycine

SDS-PAGE running buffer pH 8.3

125 mM Tris-HCl, 960 mM Glycine, 0.5% SDS

2x SDS Sample Buffer pH 8.3

100 mM Tris-HCl, pH 6.8, 4% SDS, 0.2% (w/v), Bromophenolblue, 20% (v/v) Glycerol

0.1 M PBS buffer pH 7.2 and pH 7.4

0.22 g/L NaH_2PO_4, 1.33 g/L Na_2HPO_4, 8.5 g/L NaCl

II.1.7. Enzymes, reaction kits and other chemical reagents

Table II-4. Enzymes, reaction kits and other chemical reagents.

Kit/chemical/enzyme…	Origin	Characteristics
Enzymes (*Bam*HI, *Not*I, *Esp*3I…)	Fermentas, Germany	Fast digestion
T4 DNA ligase	Fermentas, Germany	
DNTPs	Fermentas, Germany	
PageRuler™ Prestained protein ladder	Fermentas, Germany	
Agarose	Invitrogen, Germany	
QIAprep® spin Miniprep and Midi kit	Qiagen, Hilden, Germany	
QIAquick® gel extraction kit	Qiagen, Hilden, Germany	
Ni-NTA-agarose	Qiagen, Hilden, Germany	
Pwo SuperYield DNA polymerase	Roche, Germany	
ECL™ Western blotting detection reagents	GE Healthcare, UK	
MUNANA	Sigma Aldrich, USA	Substrate for N1
4-MU	Sigma Aldrich, USA	
Fetuin-crosslinked agarose	Sigma Aldrich, USA	^{Nb}H5-VLP purification
Bio-Rad protein assay reagent	Bio-Rad, Germany	
Albumin-Fraction V (BSA) and Tween 20	Serva, Germany	
Coomassie Brilliant Blue	GibcoBRL, Germany	

II.1.8. Antibiotics

All antibiotics were prepared in deionized H2O except rifampicin soluble in DMSO and sterilized using a filter (syringe filter, CA-membrane, 0.20 µm, Heinemann Labortechnik GmbH, Duderstadt, FRG). Stock and working concentration were listed in table II-5.

Table II-5. List of antibiotics.

Antibiotics	Company	Stock concentration	Working concentration
Ampicillin (Amp)	Duchefa, The Netherlands	100 g/L	100 mg/L
Carbenicillin (Carb)	Duchefa, The Netherlands	50 g/L	50 mg/L
Kanamycin (Kan)	Duchefa, The Netherlands	50 g/L	50 mg/L
Rifampicin (Rif)	Duchefa, The Netherlands	50 g/L	50 mg/L
Cefotaxim (Cef)	Duchefa, The Netherlands	200 g/L	500 mg/L

II.1.9. Antibodies

Anti-c-myc monoclonal antibody, clone 9E10, that recognizes a region (aa 408-239) of the proto-Oncogen p62 (Evan *et al*., 1985) was used for Western blot to detect recombinant proteins containing c-myc tag. Other antibodies were listed in table II-6.

Table II-6. Antibodies used for Western blot, ELISA and electron microscopy.

Antibody	Characteristics and Origin
Polyclonal rabbit antibody	Primary antibodies against whole virus NIBRG-14, provided kindly by IBT, Vietnam.
Sheep anti-Mouse IgG, Horseradish Peroxidase linked whole antibody	Secondary antibodies, GE healthcare UK limited Little Chalfont BuckinghamShire HP7 9NA UK
Donkey anti-Rabbit IgG, Horseradish Peroxidase linked whole antibody	Secondary antibodies, GE healthcare UK limited Little Chalfont BuckinghamShire HP7 9NA UK
Rabbit anti-Chicken IgY (IgG)(whole molecule), Alkaline phosphatase Conjugate	Polyclonal secondary antibodies, Sigma Aldrich, USA.
Rabbit anti-Mouse IgG (whole molecule), Alkaline phosphatase Conjugate	Polyclonal secondary antibodies, Sigma Aldrich, USA.

II.1.10. Inactivated and live influenza viruses

Inactivated virus rg A/swan/Germany/R65/2006(H5N1) was provided kindly from Institute of Molecular Biology, Friedrich-Loeffler-Institute, Federal Research Institute for Animal Health, Greifswald-Insel Riems, Germany. This is the recombinant virus which was rescued by reverse genetics composed by 7 genes from native strain (Weber *et al*., 2007) and

the modified HA containing monobasic motif at the HA cleavage site (Stech *et al*., 2008). Inactivated virus rg A/swan/Germany/R65/2006(H5N1) was used to determine hemagglutination inhibition titers with chicken and mouse sera. This virus strain was grouped into subclass 2.2.2 (Starick *et al*., 2008).

Wild-type H5N1 influenza virus: A/duck/Vietnam/TG24-01/05(H5N1) was used for virus challenge in chickens.

II.1.11. Equipment

Centrifuges

Heraeus Biofuge Fresco (Heraeus, Germany)

Thermo Scientific Sorvall Evolution RC centrifuge

Beckman Coulter Avanti® J-26 XP

Electroporation apparatus

Gene pulser™, Pulse controller unit, Extender unit and 0.2 cm or 0.4 cm cuvettes (Bio-Rad Laboratories, Hercules, CA, USA).

PCR Thermocycler

GeneAmp® PCR system 9700 (Applied Biosystem, USA)

Protein gel electrophoresis and transfer equipment

Mini PROTEAN II™ (Bio-Rad Laboratories, Hercules, CA, USA)

Mini Trans-Blot® Electrophoresis Transfer cell (Bio-Rad Laboratories, Hercules, CA, USA).

Membranes

0.2 µm cellulose acetate membrane (Sartorius Stedim, Goettingen, Germany) with membrane diameter in 47 mm.

0.22 µm polyethersulfone membrane (Corning, USA) with membrane diameter in 47 mm.

0.3 µm mixed cellulose ester membrane (Millipore, USA), membrane diameter in 47 mm.

II.2. Methods

II.2.1. General molecular cloning

Restriction enzyme reaction and ligation were performed following the manufacturer's recommendations (Fermentas, Germany. http://www.fermentas.com/en/home). DNA fragments were extracted from agarose gel by using QIAquick® gel extraction kit (Qiagen). Plasmids were prepared (miniprep or midiprep) by using solutions and columns from

QIAprep® spin Miniprep and Midi kit (Qiagen). All DNA insertions in pRTRA vectors were sequenced automatically based on the method described by Sanger and co-workers (Sanger *et al.*, 1977) using the specific primers in the table II-3. The nucleotide sequences were processed by using BioEdit Sequence Alignment Editor version 7.0.9.0 (Hall, 1999) and Lasergene® version 7 (DNASTAR inc., Madison, WI, USA).

II.2.2. Construction of expression cassettes for plant transformation

To construct the ER expression cassettes fused with 100xELP, the ectodomain H5, subunit HA1, HA2 and N1 genes were amplified by polymerase chain reaction (PCR) using primers listed in table II-2. These primers contain *Bam*HI or *Esp*3I sites at their 5'- ends. The PCR products were digested by *Bam*HI or *Esp*3I. The resulting products were inserted into pRTRA 35S-anti M1 ScFv-100xELP-HC, pRTRA USP-anti M1 ScF-100xELP-HC which were digested with *Bam*HI and dephosphorylated with Shrimp alkaline phosphatase (SAP).

In order to create the ER expression cassettes without ELP fusion, the ectodomain H5, subunit HA1, HA2 of ectodomain H5 and ectodomain N1 genes were amplified by PCR using primers in table II-2 containing *Bam*HI or *Esp*3I and *Not*I sites at their 5'- ends. The PCR products were digested by *Bam*HI or *Esp*3I and *Not*I. The resulting products were cloned into pRTRA 35S-scFv anti M1-HC, pRTRA USP-scFv anti M1-HC at *Bam*HI and *Not*I sites.

All polymerase chain reactions were performed at the same conditions: 50 µl of amplification mixture contain 0.3 µM primers (Table II-2 and Table II-7), 0.2 µM dNTPs, 2.5U Pwo SuperYield DNA polymerase, 5 µl of 10× Pwo SuperYield PCR buffer and 20 ng template. The PCR program used was denaturation at 94 °C for 30 sec, annealing at 55 °C for 50 sec and extension at 72 °C for 1 min. The program was performed with 32 cycles.

All pRTRA vectors containing the antigen expression cassettes were digested with *Hin*dIII. Then, DNA fragments were separated on an agarose gel. Fragments containing expression cassette were extracted from the agarose gel by QIAquick® gel extraction kit and cloned into the *Hin*d III sites of the plant binary vector pCB301-kan. All DNA ligation reactions were performed by using T_4 DNA Ligase according to the instructions of the manufacturer.

II.2.3. Transformation of bacteria

II.2.3.1. *Escherichia coli* transformation

Electrocompetent E. coli XL1-blue cells were prepared by the protocol described by Sambrook and Russell (Sambrook and Russell, 2006). Overnight bacterial culture was

recultured at 37 °C and 140 rpm until an OD600 value reached to 0.4-0.5. The cells were then harvested by centrifugation (3000 *g*, 15 min, 4 °C), the resulting pellet was washed one time with cold sterile H2Odd and twice with 10% (v/v) glycerol. The last pellet was resuspended in 10% (v/v) glycerol, the cell suspension was aliquoted and stored at -80 °C until use. The mixture of the electrocompetent cells and plasmids (ligation products, plasmids) was pipetted in a cold electroporation cuvette, a pulse of electricity to the cells was delivered by the Gene Pulser® (Bio-Rad Laboratories, Hercules, CA, USA) at 25 µF capacitance, 2.5 kV and 200 Ohm resistance. After the pulse, SOC medium was immediately added and cells were kept at 37 °C for 1 h. The electroporated cells were spread on LB agar medium containing appropriate antibiotics.

II.2.3.2. *Agrobacterium tumefaciens* transformation

Transformation of *A. tumefaciens* by electroporation was carried out following Mersereau's protocol (Mersereau *et al*., 1990). Transformation of the cells was performed by electroporation at the conditions described in section II.2.3.1. After the pulse, SOC medium was added and cells were shaken at 28 °C for 1 h. The electroporated cells were spead on YEB agar medium and grown at 28 °C for 48 h.

II.2.4. Transient assay in *Nicotiana benthamiana*

Agrobacteria containing the plant vector for HcPro expression were used for the transient expression of target proteins in intact leaves of *N. benthamiana* by vacuum infiltration (Kapila *et al*., 1997). HcPro is a suppressor of gene silencing that has been found to enhance remarkably the expression levels of recombinant proteins in plant cells (Conley *et al.*, 2009a; Sudarshana *et al.*, 2006). Agrobacteria harbouring shuttle vectors for expression of recombinant proteins and the plant vector for expression of HcPro were pre-cultivated separately in 2 ml of YEB medium with 50 µg/ml Kan, 50 µg/ml Carb and 50 µg/ml Rif, grown overnight at 28 °C and 140 rpm. The preculture was added to a new culture containing appropriate antibiotics. After further 24 h of cultivation, bacteria were harvested by centrifugation at 4000 *g*, 10 min, 4 °C and resuspended in infiltration buffer (10 mM 2-(N-morpholino) ethanesulphonic acid MES, 10 mM $MgSO_4$, pH 5.6). Agrobacteria harbouring shuttle vector for expression of recombinant protein and the plant vector for expression of HcPro were combined. Agrobacterium suspensions were diluted in infiltration buffer to a final OD_{600} of 1.0. *N. benthamiana* plants (six to eight weeks old) were infiltrated by completely submerging each plant in the Agrobacterium-containing cup standed inside a desiccator. Vacuum was applied during 2 min and then quickly released. The plants were then placed in

the greenhouse at 21 °C, 16 h light per day. Four days after infiltration, leaf samples were harvested and stored at -80 °C.

With the constructs under control of seed specific USP promoter, Agrobacteria bearing shuttle vectors were coinfiltrated with Agrobacteria bearing FUS3 transcription factor under control of the CaMV 35S promoter.

II.2.5. Stable transformation of *Nicotiana tabacum*

Tobacco was transformed by agroinfection following the leaf dics method described by Horsch and co-workers (Horsch *et al*., 1985). The *A. tumefaciens* C58C1 strain transformed with the pCB-kan binary vectors was grown overnight in YEB medium containing 50 µg/ml Kan, 50 µg/ml Carb and 50 µg/ml Rif. Tobacco leaf discs were submerged for 1 h in the agrobacterum culture and then plated on MS medium for another 2 days at 24 °C in the dark. Infected leaf fragments were transferred on MS medium containing 0.2 mg/L NAA, 1 mg/L BAP, 50 mg/L Kan and 500 mg/L Cef (NBKC medium). Every 10-14 days, leaf discs were transferred to the new NBKC medium until plantlets appear with 2-3 cm in length. These plantlets were transferred to MS medium containing 50 mg/L Kan (Floss and Conrad, 2010). Leaves of these plants were used for Western blot analysis to determine the antigen expression level in transgenic plants under control of the CaMV 35S promoter. Transgenic tobaccos were cultivated in greenhouse up to seed formation and ripening.

II.2.6. Western blot analysis of recombinant proteins in plants

II.2.6.1. SDS-PAGE and Western blot

Frozen leaf discs were homogenized in a Mixer Mill MM 300 (Retsch, Haan, Germany) and the resulting powder was suspended in SDS sample buffer, held at 95 °C for 10 min and then centrifuged (19,000 *g*, 30 min, 4 °C). The concentration of total soluble protein (TSP) was determined using the Bradford assay. Extracted plant proteins were separated by reducing SDS-PAGE (10% polyacrylamide) and electrotransferred to nitrocellulose membranes. After blocking with 5% (w/v) fat-free milk powder dissolved in TBS, the membranes were incubated for 2 h at room temperature with a monoclonal anti-c-myc antibody. The presence of this antibody was detected by the addition of a 1:2,000 dilution of HRP-conjugated sheep anti-mouse IgG and the signal visualized using the ECL method. Each membrane was washed three times between each step with TBS containing 0.5% w/v fat-free milk, except for the penultimate (TBS only) and final (phosphate-buffered saline, PBS)

washes. Antibodies were diluted in TBS, 5% (w/v) fat-free milk powder (Gahrtz and Conrad, 2009).

When hemagglutinins were detected by Western blot using rabbit anti serum raised against A/Vietnam/1194/2004(H5N1) as primary antibody, the HRP-conjugated donkey anti-mouse IgG antibody was subsequently used as a secondary antibody.

II.2.6.2. Blue native PAGE (BN-PAGE)

Blue native PAGE gel was performed for separation of native structure antigens following the method described by Wittig and co-workers (Wittig *et al*., 2006). Briefly, protein solutions were taken up in sample buffer (50 mM NaCl, 50 mM Imidazole, 0.5% Coomassie blue G-250, 10% glycerol, pH 7.0) and applied to a 3.5-10% polyacrylamide gradient gel (75 mM Imidazole, 500 mM 6-aminohexanoic acid, pH 7.0). The BN-PAGE gel was run in blue cathode buffer A (50 mM Tricine, 7.5 mM Imidazole, 0.02% Coomassie blue G-250, pH 7.0) and anode buffer (25 mM Imidazole, pH 7.0). Once the blue running front has moved about one-third of the whole running distance, cathode buffer A was replaced by cathode buffer A/10 (50 mM Tricine, 7.5 mM Imidazole, 0.002% Coomassie blue G-250, pH 7.0). After electrophoresis, proteins were electrotransferred to a Hybond-P polyvinylidene difluoride membranes (Millipore) in electroblotting buffer (50 mM Tricine, 7.5 mM Imidazole, pH 7.0) overnight at 18 voltage. Membrane was washed with methanol to destain Coomassie dye. Western blot using monoclonal anti-c-myc antibody was carried out (section II.2.6.1).

II.2.6.3. Cross-linking reaction

Cross-linking reaction was performed for determination of multimeric state of the purified proteins following the method described by Weldon and co-workers (Weldon *et al*., 2010). In brief, 1 µg recombinant proteins was mixed with Bis[sulfosuccinimidyl] suberate (BS3) to 5 mM final concentration and incubated for 30 min at room temperature. Cross-linking reaction was stopped by the addition of 1 M Tris-HCl pH 8.0 to a final concentration of 50 mM and incubated for 15 min at room temperature. After cross-linking, proteins were separated on a 4-10% SDS-PAGE under reducing conditions, blotted and analysed by Western blot using anti-c-myc monoclonal antibody described in detail in section II.2.6.1.

II.2.7. Kanamycin resistance assay on seedlings

100 seeds were surface-sterilized and germinated on 50 µg/ml Kan containing MS medium. Five-week-old green plantlets with several leaves were scored as resistant (R) and white ones were scored as sensitive (S). Statistical analyses were performed by the counting R

and S seeds on kan-containing selective MS media. Distribution of progeny was tested against the expected ratios using the chi-square ($\chi 2$) test, which was used to analyse segregation of the *npt II* gene in the progenies.

II.2.8. Protein purification

II.2.8.1. ITC purification of ELP fusion proteins

Frozen *N. tabacum* leaf (150 g) was ground with mortar and pestle in liquid nitrogen and homogenized in 220 ml ice-cold 50 mM Tris-HCl (pH 8.0). A Complete Protease Inhibitor Cocktail tablet (Roche, Germany) was added to the slurry, which was then cleared by centrifugation (75,600 *g*, 30 min, 4 °C) before the addition of NaCl to a final concentration of 2 M. The cold extract with 2 M NaCl was centrifuged again at 75,600 *g* for 30 min at 4 °C. The solution was then passed through a 0.22 µm polyethersulfone membrane (Corning, USA) with the temperature maintained at 4 °C, to produce a pre-treated extract.

II.2.8.1.1. Centrifugation-based ITC (cITC)

The pre-treated extract was first incubated for 30 min at 40 °C (for ^{Nt}N1-ELP, ^{Nt}H5-ELP, ^{Nb}(HpII-ELP)3 and ^{Nb}(pLI-N1-ELP)4 purification) or 55 °C (^{Nt}HA1-ELP) to induce aggregation of the antigen-ELP fusions, which were then precipitated by centrifugation (8,000 *g*, 30 min, 40 °C or 55 °C, respectively). The resulting pellet was dissolved in water at 4 °C and any insoluble matter was removed by centrifugation (15,000 *g*, 30 min, 4 °C).

II.2.8.1.2. Membrane-based ITC (mITC)

The pre-treated extract was warmed to room temperature and passed through a 0.2 µm cellulose acetate membrane using a vacuum pump (Vacuubrand, Germany). The membrane was washed twice with 2 M NaCl to remove contaminating proteins. Ice-cold Millipore-Q water was then passed through the filter to elute the protein-ELP fusions.

When ELP fusion proteins were purified by mITC from the transiently transformed *N. benthamiana* leaves, the purification procedure was adapted. 50 g of the transiently transformed *N. benthamiana* leaves were ground in liquid nitrogen and the resulting powder was homogenized in 170 ml ice-cold 50 mM Tris-HCl (pH 8.0). The plant extract was cleared by centrifugation three times (75,600 *g*, 45 min, 4 °C) before the addition of NaCl to a final concentration of 2 M. The cold extract with 2 M NaCl was centrifuged again at 75,600 *g* for 45 min at 4 °C. The resulting extract with 2 M NaCl was passed through a 0.3 µm mixed cellulose ester membrane and then through a 0.22 µm polyethersulfone membrane. This filtrate was

centrifuged again (75,600 *g*, 30 min, 4 °C). The resulting extract was used for mITC purification.

The purification efficiency of the various recombinant protein-ELP fusions was assessed by sampling the plant extract before and after the cellulose acetate filtration (mITC method), or before and after the hot centrifugation (cITC method). The samples were serially diluted to allow a semi-quantitative Western blotting-based analysis (section II.2.6.1). Band intensities were compared by image densitometry, using the totalLab TL100 software (Nonlinear Dynamics, USA).

II.2.8.2. Purification by IMAC

Four days after vacuum Agrobacterial infiltration, leaf sample (100 g) was harvested, frozen in liquid nitrogen and homogenized using a commercial blender. Total proteins were extracted in 50 mM Tris buffer (pH 8.0). The extract was clarified by centrifugation (18,000 *g*, 30 min, 4 °C) and then filtrated through paper filters. The clear extract was mixed with Ni-NTA resin agarose washed twice with water before. After mixing for 30 min at 4 °C, the mixture was applied on a chromatography column. Thereafter, the column was washed with two liters of washing buffer (50 mM NaH_2PO_4, 300 mM NaCl, 30 mM Imidazole, pH 8.0). Recombinant proteins were then eluted from the column by elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 125 mM Imidazole, pH 8.0). Protein was concentrated by an iCON™ Concentrator with a molecular-weight cutoff of 9,000 and stored at -20 °C.

II.2.8.3. Purification of virus-like particles using fetuin-linked agarose

Purification of the plant-derived VLPs was described by D´Aoust and co-workers using fetuin-cross-linked agarose (D'Aoust *et al.*, 2008). Briefly, frozen *N. benthamiana* leaves expressing H5 were extracted in the buffer containing 50 mM Tris, 50 mM NaCl, 0.04% sodium metabisulfide and homogenized by using the commercial blender. The extract was centrifuged twice at 18,000 *g*, 30 min, 4 °C. The pH of the resulting solution was adjusted to 6 using HCl 1N and then centrifuged again at 18,000 *g*, 30 min, 4 °C. The clear solution was applied on the chromatography column containing the fetuin-cross-linked agarose. The column was then washed three times with washing buffer containing 400 mM NaCl, 25 mM Tris, pH 6.0 and the bound proteins were eluted from the column by using elution buffer containing 1.5 M NaCl, 50 mM MES, pH 6.0. The protein solution was concentrated and buffer was exchanged using iCON™ concentrators with a molecular-weight cutoff of 20,000.

II.2.9. Neuraminidase activity assay

A fluorescence-based assay was used to measure NA activity, as described elsewhere (Potier *et al.*, 1979; Yongkiettrakul *et al.*, 2009). The assay was performed by adding a 10 µl aliquot of the neuraminidase sample to an equal volume of 32.5 mM MES, 4 mM CaCl2, pH 6.5. The enzymatic reaction was then initiated by the addition of 30 µl 2'-(4-methylumbelliferyl)-α-D-N-acetylneuraminic acid (Sigma, St. Louis, MO, USA). After incubation at 37 °C for 30 min, the reaction was stopped by the addition of 150 µl 100 µM glycine (pH 10.7) in 25% ethanol. The release of 4-methylumbelliferone (4-MU) was measured spectrophotometrically at 355 and 460 nm and quantification was based on a standard curve prepared with pure free 4-MU (Sigma, St. Louis, MO, USA). One unit of NA was defined as the quantity able to release 1 µM 4-MU per min at 37 °C. The % recovery of neuraminidase based on NA activity was defined as the ratio of the activity of neuraminidase recovered after mITC or cITC, to the activity of neuraminidase from plant extract before ITC.

Frozen *N. tabacum* leaf discs expressing ^{Nt}(pLI-N1-ELP)4 were homogenized in a Mixer Mill MM 300 (Retsch, Haan, Germany) and the resulting powder suspended in 20 mM Tris, 4 mM $CaCl_2$, pH 6.5. The extract was centrifuged at 19,000 *g*, 30 min, 4 °C. The resulting plant extract was used to measure ^{Nt}(pLI-N1-ELP)4 activity.

II.2.10. Mouse vaccination

6–8 weeks old male BL6 (C57/Black6J) mice (ten per group, Charles River Laboratories, Research Models and Services, Germany GmbH) were immunized subcutaneously with ^{Nt}H5, ^{Nb}(H5pII)3, ^{Nt}H5-ELP and ^{Nb}(H5pII-ELP)3 on day 0, 14, 21 and 35 with doses of 10 µg and 50 µg per immunization. The antigens were formulated with 10 µg of Complete Freund's adjuvant in the first immunization and with 10 µg of Incomplete Freund's adjuvant (Sigma, St. Louis, MO, USA) in the following immunization. In control groups, mice received PBS plus adjuvants. Mice were then retro-orbitally bled one week after the 3rd and 4th immunization. Mouse sera were collected individually for hemagglutination inhibition (HI) and ELISA tests.

When abISCO®-100 (abISCO) (Isconova, Uppsala, Sweden) comprising soponins, cholesterol and phospholipid molecules was used as the adjuvant, mice were vaccinated subcutaneously with only trimer antigens: ^{Nb}(H5pII)3 and ^{Nb}(H5pII-ELP)3 with the dose of 10 µg per immunization at day 0, 14 and 28. One week after each immunization, mouse sera were collected individually for HI and ELISA tests.

II.2.11. Hemagglutination test and hemagglutination inhibition assay

II.2.11.1. Collection and preparation of rooster red blood cells

For hemagglutination test and hemagglutination inhibition test, blood (8 ml) from wing vein of roosters which were not vaccinated for Newcastle disease virus or other pathogens were collected in a sterile bottle containing 3.2% sodium citrate (2 ml). The bottle was rotated gently to mix thoroughly. An equal volume of 0.1 M PBS, pH 7.2 was added and the suspension was centrifuged at 3000 g for 5 min to pellet erythrocytes. The erythrocytes were washed twice with 0.1 M PBS, pH 7.2. 198 ml of 0.1 M PBS, pH 7.2 were added to 2 ml of packed red blood cells to get a final erythrocyte concentration of 1%.

II.2.11.2 Hemagglutination test

Hemagglutination assays were performed with different antigens, viruses and VLPs. The assay is based on the OIE protocol (OIE, 2004). Briefly, 25 µl of antigen were added into the first well of a plastic V-bottom microtiter plate containing 25 µl of PBS. Two-fold serial dilution was made across the entire row. A further 25 µl of PBS were added and mixed thoroughly and then, 25 µl of 1% red blood cells (RBC) were added. Results were read after plates were incubated at 25 °C for 30 min. The endpoint dilution that causes a complete hemagglutination was defined as one hemagglutination unit (HAU).

II.2.11.3 Hemagglutination inhinbition (HI) assay

Hemagglutination inhibition assays were performed by a standard procedure based on the OIE protocol (OIE, 2004). Mouse sera were pre-treated with receptor destroying enzyme, RDR II (Denka Seiken Co. Ltd, Tokyo, Japan) for 16 h at 37 °C (1 volume of serum : 3 volume of RDE II). 25 µl of serum were added into the first well of a plastic V-bottom microtiter plate containing 25 µl of PBS. Serial two dilution was made across the entire row. 4 HAU in 25 µl of inactivated virus rg A/swan/Germany/R65/2006(H5N1) or the purified virus-like particles (^{Nb}H5-VLP) from A/Hatay/2004(H5N1) strain were added and incubated at 25 °C for 30 min. 25 µl of 1% RBC were then added to each well and incubated for 30 min at 25 °C. HI titer was read as the reciprocal of the highest dilution of serum that inhibited complete hemagglutination.

II.2.12. ELISA

II.2.12.1. Antigen ELISA

For testing mouse sera, microtiter plates (ImmunoPlate Maxisorp, Nalgen Nunc International, Roskilde, Denmark) were coated with 100 µl of 3 µg/mL recombinant antigen in PBS and incubated overnight at 4 °C. After blocking with 3% (w/v) bovine serum albumin (BSA), 0.05% (v/v) Tween20 in PBS (PBST) for 2 h, 100 µl of the specific dilution ($2x10^{-4}$) were applied and incubated at room temperature for 1.5 h. Plates were washed 5 times with PBST, 100 µl of a rabbit anti-mouse IgG alkaline phosphatase conjugate diluted (2000 times) in 1% (w/v) BSA in PBST were then added. The enzymatic substrate, p-nitrophenyl phosphate (pNPP) in 0.1 M diethanolamine-HCl (pH 9.8), was added and the absorbance signal was measured at 405 nm after a 1 h incubation at 37 °C.

For testing chicken sera, microtiter plates (ImmunoPlate Maxisorp, Nalgen Nunc International, Roskilde, Denmark) were coated with 100 µl of 10 µg/mL recombinant antigen in PBS and incubated overnight at 4 °C. After blocking with 3% (w/v) BSA, 0.05% (v/v) Tween20 in PBS (PBST) for 2 h, 100 µl of the specific dilution (2x10-4) were applied and incubated at room temperature for 1.5 h. Plates were washed 5 times with PBST, 100 µl of a rabbit anti-chicken IgY antibodies-conjugated alkaline phosphatase diluted (35 000 times) in 1% (w/v) BSA in PBST were then added. The enzymatic substrate, pNPP in 0.1 M diethanolamine-HCl (pH 9.8), was added and the absorbance signal was measured at 405 nm after a 1 h incubation at 37 °C.

II.2.12.2. Competitive ELISA

Microtiter plates (ImmunoPlate Maxisorp, Nalgen Nunc International, Roskilde, Denmark) were coated with 3 µg/mL recombinant antigen in PBS and incubated overnight at 4 °C. After blocking with 3% (w/v) BSA, 0.05% (v/v) Tween20 in PBS (PBST) for 2 h, various concentrations of pre-determined quantities of recombinant antigen mixed with specific dilution of mouse serum ($2x10^{-4}$) in 1% (w/v) BSA in PBST were added to each well, and the plates incubated for 1.5 h at 25 °C. Antigen-antibody complexes were visualized by treatment with rabbit anti-mouse IgG alkaline phosphatase conjugate diluted in 1% (w/v) BSA in PBST. The enzymatic substrate pNPP was added and the absorbance was measured at 405 nm after a 1 h incubation at 37 °C.

II.2.13.Virus challenge in chicken

Virus challenge in chickens was conducted by Dr. Jutta Veits at Institute of Molecular Biology, Friedrich-Loeffler-Institute, Federal Research Institute for Animal Health, Greifswald-Insel Riems, Germany.

6 weeks old white Leghorn chickens (ten per group, Lohmann Tierzucht) were immunized intramuscularly with ^{Nt}H5-ELP and ^{Nt}HA1-ELP with doses of 10 µg and 100 µg per animal. The antigens were formulated with Complete Freund's adjuvant (Sigma, St. Louis, MO, USA). In control group, chickens received PBS plus Complete Freund's adjuvant. On day 18, chicken sera were collected individually for HI tests and ELISA. 21 days after the first immunization, all immunized chickens and negative control group were challenged by oculonasal with A/duck/Vietnam/TG24-01/05(H5N1) virus strain at a dose of 10^6 egg infectious doses (10^6 EID_{50}).

II.2.14. Electron microscopy

Electron microscopy experiment was conducted by Gerd Hause at Biocenter, Microscopy Unit, University of Halle-Wittenberg, Halle, Germany.

The negatively stained samples (purified ^{Nb}H5-VLP) were prepared by spreading 3 µl of the dispersion onto a Cu grid coated with a formvarfilm. After 1 min of adsorption, excess liquid was blotted off with filter paper. After air drying for 10 seconds and washing with water (3 times for 1 minute) the grids were placed on a droplet of 1% aqueous uranyl acetate and drained off after 1 min. The dried specimens were examined with a Zeiss EM 900 transmission electron microscope at an acceleration voltage of 80 kV. Electron micrographs were taken with a slow scan camera (Variospeed SSCCD camera SM-1k-120, TRS, Moorenweis, Germany).

III. Results

The objectives of this thesis are: (1) to express the recombinant hemagglutinin (H5) and neuraminidase (N1) as ELP fusion proteins in both leaves and seeds of tobacco plants, (2) to optimize an ITC-based protocol for purification of ELP fusion influenza proteins and (3) to characterize structure, functionality and immunogenicity of the influenza antigens.

III.1. Construction of expression cassettes for plant transformation

III.1.1. Generation of basic vectors containing 6x histidine and c-myc tags

The basic pRTRA vectors contained the cassettes for expression of tuberculosis antigens (TBAG or TBAG-ELP) under the transcriptional control of the CaMV 35S promoter (Floss *et al.*, 2010a). The other basic vectors contained expression cassettes for the single chain variable fragment (scFv) anti oxa, scFv anti oxa fusion with 100xELP (Scheller *et al.*, 2004; Scheller *et al.*, 2006) under control of the seed specific USP promoter. The expression cassettes in basic vectors possessed DNA sequences coding for (1) the legumin B4 signal peptide (Bäumlein *et al.*, 1986) and the KDEL signal at the N and C-terminus, respectively, for endoplasmic reticulum retention, (2) the c-myc tag (EQKLISEEDLN) that is recognized by the 9E10 antibody (Evan *et al.*, 1985) and used for the detection of recombinant proteins and (3) TBAG or scFv anti oxa and (4) 100xELP (Scheller *et al.*, 2004). These expression cassettes which do not contain a DNA sequence coding for 6x histidine are presented in Fig. III-1A.

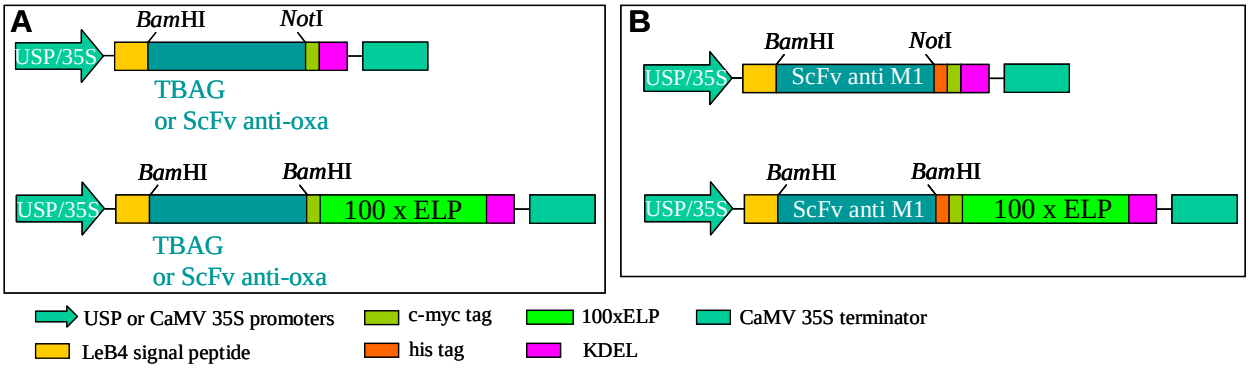

Figure III-1. Expression cassettes in basic vectors. (A) Expression cassettes in basic vectors without his tag. TBAG: tuberculosis antigens; scFv anti oxa: single chain variable fragment anti oxazolone. (B) Expression cassettes in basic vectors with his tag. ScFv anti M1: single chain variable fragment against influenza M1 protein. This scFv specific for influenza M1 was screened by using human single framework scFv phage display libraries, Tomlinson A+B (Tomlinson, T., MRC, Centre for Protein Engineering, Cambridge, UK).

To facilitate purification of recombinant proteins by immobilized metal ion affinity chromatography (IMAC), new expression cassettes that contain an additional DNA sequence coding 6x histidine were generated. The gene encoding scFv anti M1 was amplified from

pRTRA 35S-scFv anti M1 plasmid (unpublished data) using extended primers encoding 6x hisditine (HIS-c-myc-Esp3I-F and ELP-HIS-c-myc-Esp3I-R (Table II-2 and Table III-1)). The resulting PCR products were inserted into *Bam*HI opened pRTRA 35S-TBAG-100xELP and pRTRA USP-scFv anti oxa-100xELP. The resulting vectors were shown in Fig. III-1B and termed as pRTRA USP/35S-scFv anti M1-100xELP-HC. These basic vectors contain open reading frames encoding LeB4 signal peptide, scFv anti M1, 100xELP, 6x his tag, c-myc tag and the KDEL ER retention signal.

The PCR products using extended primers encoding 6x histidine (HIS-c-myc-Esp3I-F and His-c-myc-Esp3I-R (Table II-2 and Table III-1) were cloned into *Bam*HI, *Not*I digested pRTRA 35S-TBAG and pRTRA USP-scFv anti oxa. The resulting vectors were shown in Fig. III-1B and termed as pRTRA USP/35S-scFv anti M1-HC. These basic vectors contain open reading frames encoding LeB4 signal peptide, scFv anti M1, 6x his tag, c-myc tag and the KDEL ER retention signal.

ScFv anti M1 was replaced by neuraminidases and hemagglutinins either at (*Bam*HI, *Not*I) or at (*Bam*HI, *Bam*HI) to generate constructs for expression of influenza antigens that were fused with 0xELP or with 100xELP, respectively.

III.1.2. Generation of vectors for expression of the ectodomains of H5 and N1

Transmembrane domains of H5 at the C-terminus and N1 at the N-terminus and their ectodomains are shown in Fig. III-4A and 4B, respectively. To obtain truncated hemagglutinin and neuraminidase, the ectodomain H5, the subunit HA1, HA2 of the ectodomain H5 and the ectodomain N1 genes were amplified by PCR using respective specific primers listed in table III-1. The PCR products were digested with *Bam*HI or *Esp*3I and inserted into pRTRA 35S-scFv anti M1-100xELP-HC and pRTRA USP-scFv anti M1-100xELP-HC (Fig. III-1B), which were pre-digested with *Bam*HI and dephosphorylated with SAP. The resulting vectors containing expression cassettes were checked with *Bam*HI and verified by sequencing. Only *Hin*dIII fragments containing expression cassettes were selected and inserted into the pCB301 vector that was pre-treated with *Hin*dIII to generate binary vectors. Protein products of these constructs were H5-ELP, HA1-ELP, HA2-ELP and N1-ELP (Fig. III-3A). They were fused with 100xELP, his tag and c-myc tag.

To generate ER expression cassettes without 100xELP, the ectodomain H5, the subunit HA1, HA2 of the ectodomain H5 and the ectodomain N1 genes were amplified by PCR using specific primers (Table III-1) containing *Bam*HI or *Esp*3I and *Not*I sites at their 5'- ends. The

PCR products were digested separately using *Bam*HI or *Esp*3I and *Not*I and cloned into pRTRA 35S-scFv anti M1-HC and pRTRA USP-scFv anti M1-HC at *Bam*HI and *Not*I sites. The protein products of these constructs were H5, HA1, HA2 and N1. They contained both 6x his and c-myc tags (Fig. III-3A).

Table III-1. Primers and amplicons. Sequences were written in 5' to 3' direction. F: forward primer; R: reverse primer; NA: neuraminidase; HA: hemagglutinin; M1: influenza matrix protein 1; HA1 and HA2: subunit 1 and 2 of H5 protein; scFv: single chain fragment variable antibody.

Forward primer	Reverse primer	Amplicon	enzyme compatibility
HIS-c-myc-Esp3I-F	ELP-HIS-c-myc-Esp3I-R	ScFv anti M1	*Bam*HI
HIS-c-myc-Esp3I-F	His-c-myc-Esp3I-R	ScFv anti M1	*Bam*HI+*Not*I
NA-ELP-Esp3I-F	NA-ELP-Esp3I-R	Ectodomain N1	*Bam*HI
NA-ELP-Esp3I-F	NA-NotI-R	Ectodomain N1	*Bam*HI+*Not*I
NA-pLI-ELP-Esp3I-F	NA-pLI-ELP-Esp3I-R	pLI-N1	*Bam*HI
NA-pLI-SalI-F	NA-NotI-R	Ectodomain N1	*Sal*I+*Not*I
HA-Bam-F	HA-Bam-R	Ectodomanin H5	*Bam*HI
HA-Bam-F	HA-NotI-R	Ectodomanin H5	*Bam*HI+*Not*I
HA-Bam-F	HA0-NotI-R	Full H5	*Bam*HI+*Not*I
HA-Bam-F	VLP-HA-TAG-NotI-R	Full H5 with TAG	*Bam*HI+*Not*I
HA-Bam-F	opHA1-Bam-R	HA1	*Bam*HI
HA-Bam-F	opHA1-NotI-R	HA1	*Bam*HI+*Not*I
opHA2-ELP-Bam-F	opH2-Bam-R	HA2	*Bam*HI
opHA2-ELP-Bam-F	HA-NotI-R	HA2	*Bam*HI+*Not*I
HA-Bam-F	HApII-pspOMI-R	Ectodomain H5pII	*Bam*HI+*Not*I
HA-Bam-F	HApII-ELP-Bam-R	Ectodomain H5pII	*Bam*HI

III.1.3. Generation of vectors for expression of multimeric influenza antigens

To express the ectodomain of the hemagglutinin and neuraminidase as their oligomeric proteins, trimeric motif (GCN4-pII or isoleucine zipper with amino acid sequence: RMKQIEDKIEEILSKIYHIENEIARIKKLIGER) and tetrameric mofif (GCN4-pLI with amino acid sequence: RMKQIEDKLEEILSKLYHIENELARIKKLLGER) (Harbury *et al.*, 1993) were fused to the C-terminus of the ectodomain H5 and the N-terminus of the ectodomain N1, respectively. The resulting vectors and their protein products are shown in Fig. III-3C.

To this end, the DNA fragment (Fig. III-2A) encoding trimerization motif GCN4-pII and the tetramerization motif GCN4-pLI was extracted from digested pMA vectors by *Bam*HI and *Not*I, then inserted into pRTRA 35S-scFv anti M1-HC and pRTRA USP-scFv anti M1-HC

(Fig. III-1B). The resulting pRTRA 35S/USP-pLI-pII-HC vectors contained the trimerization and the tetramerization motives as well as his and c-myc tags (Fig. III-2B).

To generate constructs for expression of N1 as a tetramer, the N1 PCR product, amplified using specific primers (Table II-2 and Table III-1), was cloned into pRTRA 35S/USP-pLI-pII-HC opened by *Sal*I and *Not*I. The resulting pRTR 35S/USP-pLI-N1-HC (Fig. III-3C) was used as a template to amplify a pLI-N1 which was inserted into pRTRA 35S-scFv anti M1-100xELP-HC and pRTRA USP-scFv anti M1-100xELP-HC at *Bam*HI. The resulting pRTRA 35S-pLI-N1-100xELP-HC and pRTRA USP-pLI-N1-100xELP-HC vectors were expected to express the (pLI-N1-ELP)4 tetramer under control of the CaMV 35S and the seed specific USP promoters, respectively (Fig. III-3C).

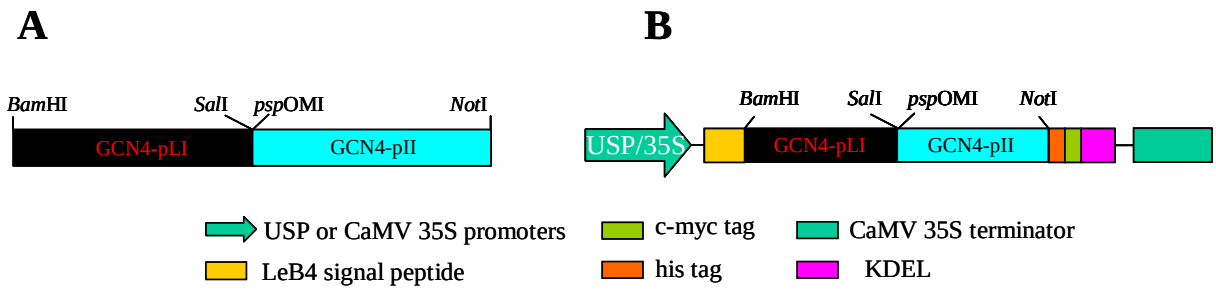

Figure III-2. GCN4-pII (pII) trimerization and GCN4-pLI (pLI) tetramerization motives. This fragment was synthesized by Geneart GmbH, Germany and cloned into pMA vector (A) and pRTRA vectors (B) at *Bam*HI and *Not*I sites.

In order to generate constructs for expression of H5 as a trimer, the H5 PCR product, amplified using specific primers (Table II-2 and Table III-1), was cloned into pRTRA 35S/USP-pLI-pII-HC opened by *Bam*HI and *psp*OMI. The resulting pRTR 35S/USP-H5-pII-HC vectors (Fig. III-3C) were used as a template to amplify a H5-pII which was inserted into pRTRA 35S-scFv anti M1-100xELP-HC and pRTRA USP-scFv anti M1-100xELP-HC at *Bam*HI sites. The resulting pRTRA 35S-H5-pII-100xELP-HC and pRTRA USP-H5-pII-100xELP-HC vectors are expected to express the (H5-ELP)3 trimer under control of the CaMV 35S and seed specific promoters, respectively (Fig. III-3C).

DNA sequence coding full H5 except its signal peptide (Fig. III-4A) was amplified by PCR and then cloned into pRTRA 35S-scFv anti M1-HC at *Bam*HI and *Not*I sites. If the reverse primer (VLP-HA-TAG-NotI-R, (Table II-2 and Table III-1) containing stop codon was used, a protein product of the resulting construct was termed as the H5-VLP (virus-like particle) (Fig. III-3B). This protein did not contain the KDEL ER retention signal and any tags because the stop codon (TAG) was inserted at the C-terminus. The protein was expected to form VLPs. When the reverse primer (HA0-NotI-R in Table II-2 and III-1) was used in PCR,

the resulting vector was expected to produce a protein named as a HA0ER. This hemagglutinin contained his and c-myc tag as well as the KDEL RE retention signal (Fig. III-3B).

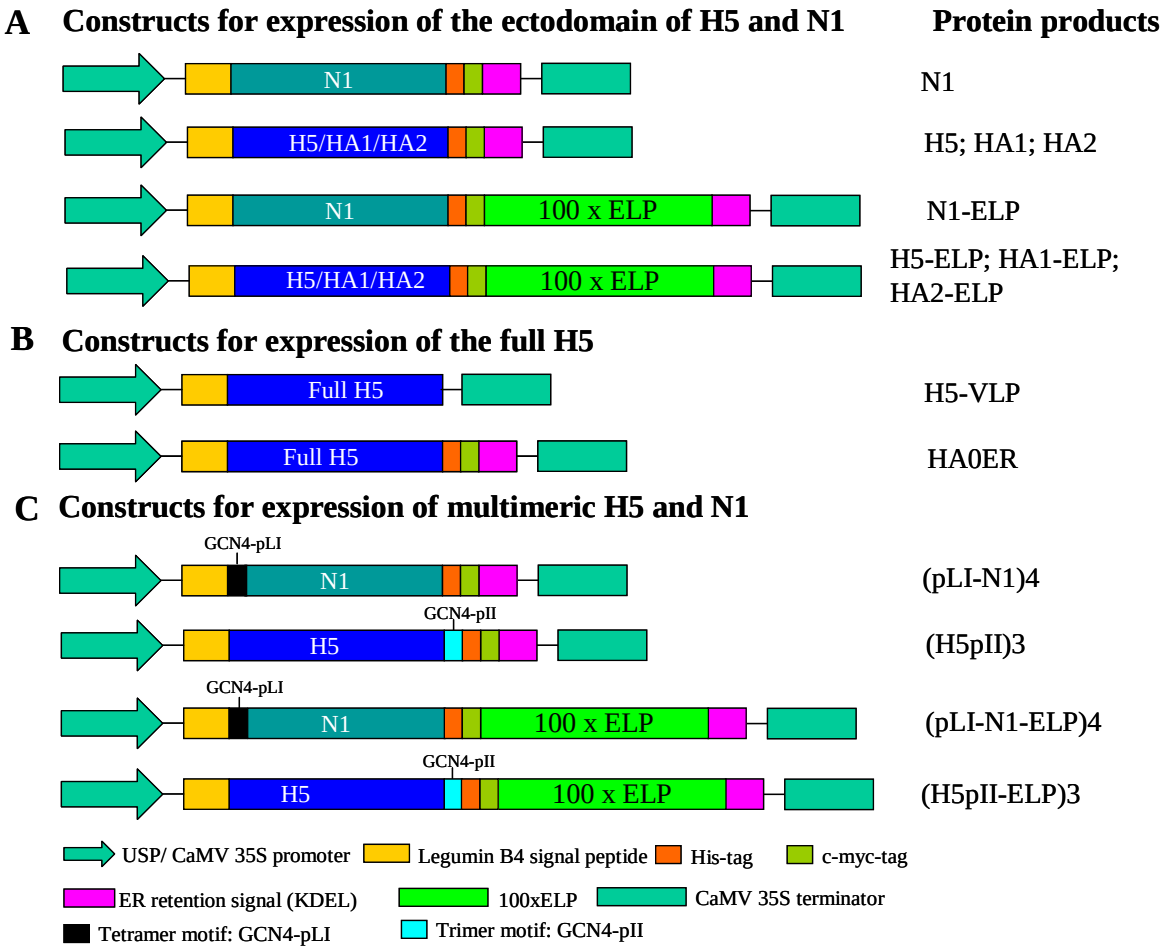

Figure III-3. Expression cassettes of hemagglutinins and neuraminidases. H5: ectodomain of the hemagglutinin subtype 5; HA1: the subunit 1 of the hemagglutinin subtype 5; HA2: the subunit 2 of the hemagglutinin subtype 5; N1: ectodomain of the neuraminidase subtype 1; Full H5: full length of hemagglutinin including transmembrane and cytoplasmic tail except signal peptide.

III.2. Transient expression of H5 and N1 in plants

The transient expression system has emerged as a rapid and useful method to analyse genes and gene products in plants. This is feasible method for testing accumulation of the foreign proteins in plant cells before processing to develop stably transformed plants. Therefore, expression of H5 and N1 proteins in *N. benthamiana* by the transient expression assay was verified carefully to select suitable constructs which could express target protein at the highest level before generating of transgenic plants.

III.2.1. Optimization of hemagglutinin and neuraminidase expression

Hemagglutinin, a transmembrane type I, contains its signal peptide at the N-terminus, transmembrane domain and cytoplasmic tail at the C-terminus (Veit and Thaa, 2011) (Fig. III-4A).

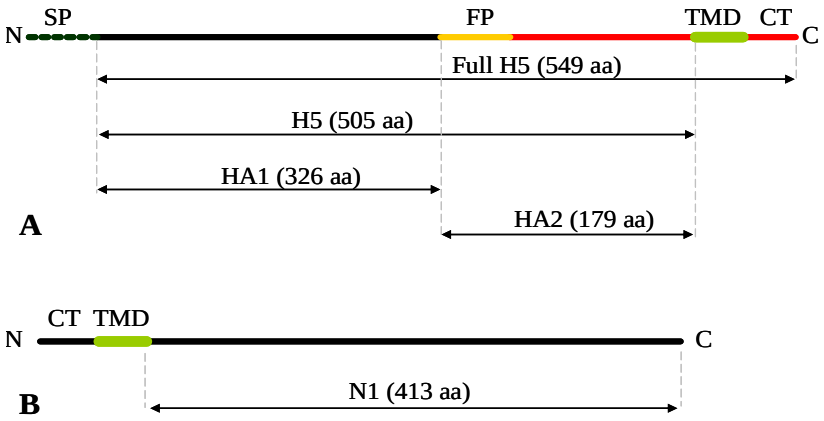

Figure III-4. Primary structure of influenza hemagglutinin and neuraminidase. (A) Hemagglutinin. (B) Neuraminidase. N: amino-terminus; C: carboxyl-terminus; SP: hemagglutinin signal peptide with 16 amino acids in length; S-S: di-sulphide bond; FP: fusion peptide; TMD: transmembrane domian; CT: cytoplasmic tail; H5 full: full length of hemagglutinin including transmembrane, cytoplasmic tail except its signal peptide; H5: ectodomain of the hemagglutinin subtype 5; HA1 and HA2: the subunit 1 and 2 of the hemagglutinin subtype 5; N1: ectodomain of the neuraminidase subtype 1.

In order to determine which part of hemagglutinin can be accumulated in tobacco plants, five different variants (H5, HA1, HA2, HA0ER and H5-VLP) of hemagglutinin protein (Fig. III-4A, 3A and 3B) were expressed in *N. benthamiana* by the transient assay under control of the CaMV 35S promoter (section II.2.4). Four recombinant proteins (H5, HA1, HA2, HA0ER) contained 6x his, c-myc tags and the KDEL at C-terminus providing IMAC purification, Western blot detection and the endoplasmic reticulum (ER) retention, respectively (Fig. III-3A and 3B), whereas H5-VLP did not contain any tags (Fig. III-3B).

Four days after agroinfiltration, proteins were solubilized in SDS sample buffer, separated in SDS-PAGE and analysed by Western blot (section II.2.6.1). Immunoblot results are shown in Fig. III-5A. No signal was detected for ^{Nb}HA0ER (lane 1, Fig. III-5A) containing the TMD, CT and KDEL, while hemagglutinin variants (^{Nb}H5, ^{Nb}HA1 and ^{Nb}HA2) without TMD and CT were clearly detectable by Western blot using anti-c-myc monoclonal antibody. When the stop codon (TAG) was introduced after the CT to generate a full hemagglutinin (^{Nb}H5-VLP) containing TMD, CT but lacking 6x his, c-myc tags and the KDEL, this hemagglutinin was expressed and recognized by rabbit anti serum raised against

A/Vietnam/1194/2004(H5N1) (Fig. III-5B). The result in Fig. III-5A shows that the hemagglutinins were accumulated with high expression level in the ER when the TMD and CT were removed. The expression of the ectodomain H5, the subunit HA1 and HA2 of hemagglutinin H5 was further tested in fusion with ELP in Fig. III-6, while the ^{Nb}H5-VLP was supposed to be transported to the plasma membrane *via* the secretory pathway. The purification and structural and functional characterization of this protein have been shown in Fig. III-15, 27 and 28.

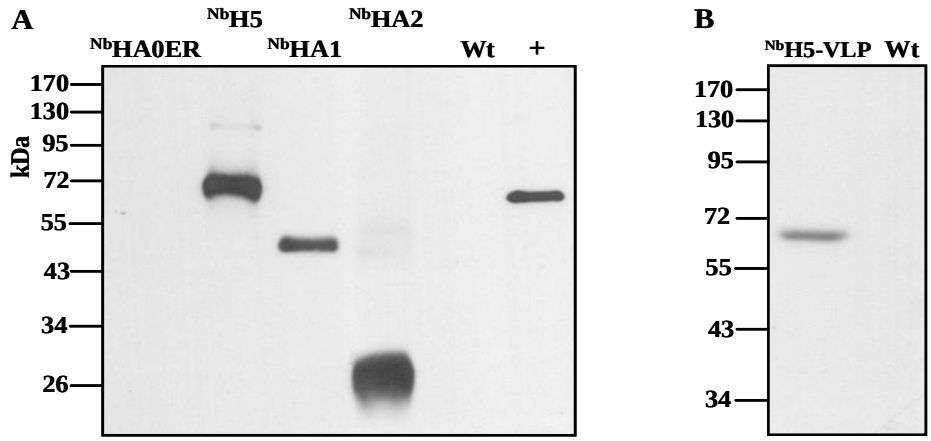

Figure III-5. Optimizing expression of hemagglutinin in *N. benthamiana*. (A) Recombinant proteins were detected by Western blot using anti-c-myc monoclonal antibody. Different lengths of the hemagglutinin from the (A/Hatay/2004(H5N1) strain were transiently expressed in *N. benthamiana*. ^{Nb}HA0ER: full length of hemagglutinin including TMD, CT and the KDEL ER retention signal; H5: the ectodomain of the hemagglutinin subtype 5; ^{Nb}HA1 and ^{Nb}HA2: the subunit 1 and 2 of the hemagglutinin subtype 5; C+: 1ng ^{Nt}anti-hTNFα-VHH-ELP used as a Western blot standard. (B) Recombinant protein was detected by Western blot using polyclonal rabbit antibodies against A/Vietnam/1194/2004(H5N1). ^{Nb}H5-VLP: full hemagglutinin including its transmembrane and cytoplasmic tail but not signal peptide. Nb: indicates that recombinant proteins were expressed transiently in *N. benthamiana*. 30 µg of TSP were loaded on SDS-PAGE.

The influenza neuraminidase protein, a transmembrane type II, contains cytoplasmic tail and transmembrane at the N-terminus (Fig. III-4B). In order to express neuraminidase in plant cells, the ectodomain of neuraminidase () was constructed and expressed in plants as the neuraminidase ELP fusion or neuraminidases without ELP (Fig. III-3A and C).

III.2.2. Transient expression of H5 and N1 in tobacco leaves

III.2.2.1. Transient expression of H5 and N1 under control of the CaMV 35S promoter

Under control of the CaMV 35S promoter, agroinfiltration was carried out to introduce Agrobacteria into intact leaves of *N. benthamiana* by vacuum. To achieve high level expression of the recombinant proteins, Agrobacteria containing binary vector for expression

of HcPro suppressor of gene silencing were coinfiltrated with Agrobacteria carrying binary vector expression for influenza hemagglutinins and neuraminidases.

Four days after infiltration, total soluble proteins were extracted and analysed in SDS-PAGE. The target proteins were detected by Western blot using anti-c-myc monoclonal antibody. The results are presented in Fig. III-6. Western blot analyses indicate that all hemagglutinin protein variants were expressed in plants (Fig. III-6A).

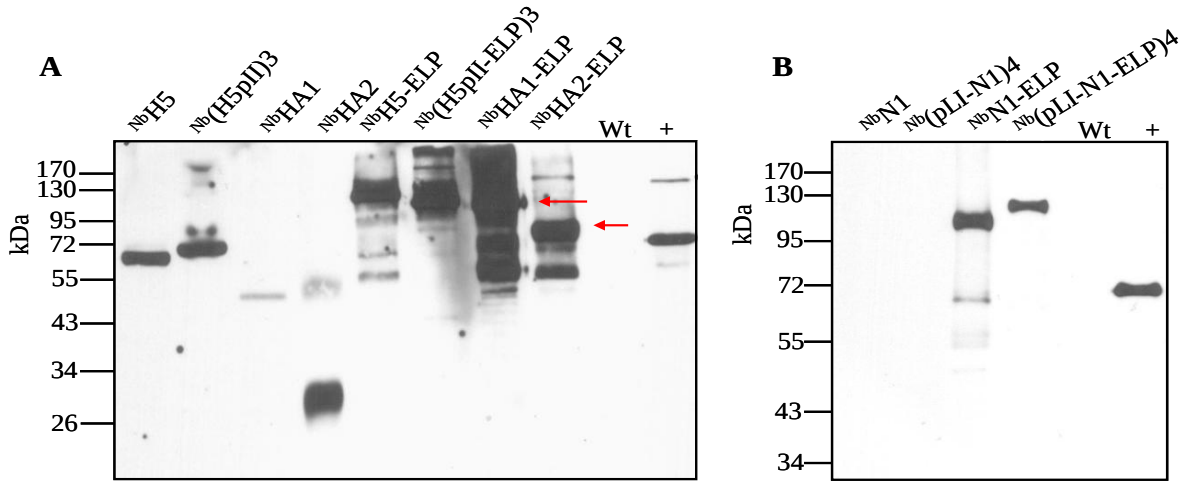

Figure III-6. Transient expression of hemagglutinin and neuraminidase proteins in *N. benthamiana* under control of the CaMV 35S promoter. Recombinant hemagglutinins (A) and neuraminidases (B) were separated by 10% SDS-PAGE, blotted and detected by anti-c-myc monoclonal antibody. 30 µg TSP from leaves expressing antigens alone and 3 µg TSP from leaves expressing ELPylated proteins were loaded. ^{Nb}H5 and ^{Nb}(H5pII)3: the ectodomain of the hemagglutinin subtype 5 without and with GCN4-pII; ^{Nb}HA1 and ^{Nb}HA2: the subunit 1 and 2 of the hemagglutinin subtype 5; ^{Nb}H5-ELP and ^{Nb}(H5pII-ELP)3: the ectodomain of the hemagglutinin subtype 5 fused to ELP, without and with GCN4-pII, respectively; ^{Nb}HA1-ELP and ^{Nb}HA2-ELP: the subunit 1, 2 fused to ELP, respectively; ^{Nb}N1 and ^{Nb}(pLI-N1)4: ectodomain of the neuraminidase without and with GCN4-pLI; ^{Nb}N1-ELP and ^{Nb}(pLI-N1-ELP)4: ectodomain of the neuraminidase fused to ELP, without and with GCN4-pLI; +: 1ng ^{Nt}anti-hTNFα-VHH-ELP used as a Western blot standard; Wt: wild type. Nb: recombinant proteins were expressed transiently in *N. benthamiana*.

In case of neuraminidase, the immunogical signal was only detected in extracts from leaves expressing ^{Nb}N1-ELP and ^{Nb}(pLI-N1-ELP)4 (Fig. III-6B), while ^{Nb}N1 and ^{Nb}(pLI-N1)4 were not detectable. In reducing SDS-PAGE, the monomeric ^{Nb}H5 and trimeric ^{Nb}(H5pII)3 proteins migrated as a single polypeptide of approximately 60.8 and 65.2 kDa proteins, respectively. The bigger molecular weight of ^{Nb}(H5pII)3 was the result of the isoleucine zipper fusion. This was also observed with the ^{Nb}N1-ELP and ^{Nb}(pLI-N1-ELP)4 where the molecular weight of the ^{Nb}(pLI-N1-ELP)4 was bigger than that of the ^{Nb}N1-ELP. The molecular weights of all recombinant proteins without additional glycosylation are demonstrated in table III-2.

III.2.2.2. Transient expression of H5 and N1 under control of the USP promoter

To test the expression of hemagglutinin and neuraminidase proteins under control of the seed specific USP promoter in *N. benthamiana* leaves, the seed specific transcription factors were required. In this experiment, the FUS3 transcription factor was used. The USP controlled constructs and the construct containing the CaMV 35S controlled FUS3 transcription factor (Bäumlein *et al*., 1994) were coinfiltrated into intact leaves of *N. benthamiana* by using the respective Agrobacteria.

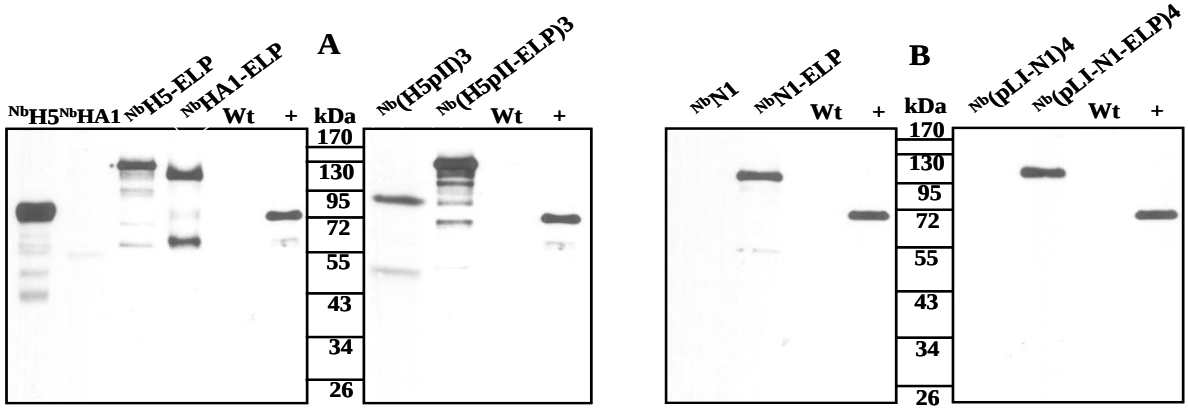

Figure III-7. Transient expression of hemagglutinin and neuraminidase proteins in *N. benthamiana* under control of the seed specific USP promoter. (A) Recombinant hemagglutinin protein variants. (B) Recombinant neuraminidase protein variants. 30 µg TSP from leaves expressing antigens alone and 3 µg TSP from leaves expressing ELPylated proteins were loaded. Proteins were separated by 10% SDS-PAGE, blotted and detected by anti-c-myc monoclonal antibody; ^{Nb}H5 and ^{Nb}(H5pII)3: the ectodomain of the hemagglutinin subtype 5 without and with GCN4-pII; ^{Nb}HA1 and ^{Nb}HA2: the subunit 1 and 2 of the hemagglutinin subtype 5; ^{Nb}H5-ELP and ^{Nb}(H5pII-ELP)3: the ectodomain of the hemagglutinin subtype 5 fused to ELP, without and with GCN4-pII, respectively; ^{Nb}HA1-ELP and ^{Nb}HA2-ELP: the subunit 1, 2 fused to ELP, respectively; ^{Nb}N1 and ^{Nb}(pLI-N1)4: ectodomain of the neuraminidase without and with GCN4-pLI; ^{Nb}N1-ELP and ^{Nb}(pLI-N1-ELP)4: ectodomain of the neuraminidase fused to ELP, without and with GCN4-pLI; +: 1ng ^{Nt}anti-hTNFα-VHH-ELP used as a Western blot standard; Wt: wild type. Nt and Nb: recombinant proteins were expressed in transgenic *N. tabacum* and in *N. benthamiana*, respectively.

The expression of recombinant proteins under control of the USP promoter was confirmed by Western blot analysis using anti-c-myc monoclonal antibody. The Western blot results in Fig. III-7A demonstrate that hemagglutinin protein variants (^{Nb}H5, ^{Nb}H5-ELP, ^{Nb}(H5pII)3, ^{Nb}(H5pII-ELP)3, ^{Nb}HA1 and ^{Nb}HA1-ELP) were successfully expressed in *N. benthamiana*. Interestingly, the addition of the isoleucine zipper at the C-terminal H5 or between H5 and ELP did not affect the expression levels of ^{Nb}(H5pII)3 and ^{Nb}(H5pII-ELP)3 in comparison to the ^{Nb}H5 and ^{Nb}H5-ELP. The expression level of the ^{Nb}(H5pII-ELP)3 was even higher than that of the ^{Nb}H5-ELP (Fig. III-7A). Only the ^{Nb}N1-ELP and ^{Nb}(pLI-N1-ELP)4 were

immunogically visible, while the expression levels of the ^{Nb}N1 and the ^{Nb}(pLI-N1)4 are so low that immunogical signals were not detectable (Fig. III-7B). All protein bands in Western blot (Fig. III-6 and 7) corresponded to the expected protein sizes (Table III-2). Under reducing SDS-PAGE, ^{Nb}H5, ^{Nb}(H5pII)3, ^{Nb}(H5pII-ELP)3 and ^{Nb}(pLI-N1-ELP)4 were observed as proteins with the molecular weight of approximately 60.8, 65.2, 106 and 94 kDa, respectively. Furthermore, the expression levels of 100xELP fuison antigens were significant higher than those of recombinant proteins without 100xELP.

Table III-2. Molecular weights of recombinant proteins. Molecular weights of proteins based on their amino acid compositions were determined by Lasergene software without glycan attachments. HA1 and HA2: the subunit 1 and 2 of the hemagglutinin subtype 5; H5 and $(H5pII)_3$: monomer and trimer of the ectodomain hemagglutinin subtype 5, respectively; N1 and $(pLI\text{-}N1)_4$: monomer and tetramer of the ectodomain neuraminidase subtype 1, respectively; H5-VLP: the full hemagglutinin subtype 5 that could form virus-like particles; HA1-ELP and HA2-ELP: the subunit 1 and 2 of the hemagglutinin subtype 5 fused to ELP, respectively; H5-ELP and $(H5pII\text{-}ELP)_3$: monomer and trimer of the ectodomain hemagglutinin subtype 5 fused to ELP; N1-ELP and $(pLI\text{-}N1\text{-}ELP)_4$: monomer and tetramer of the ectodomain neuraminidase subtype 1 fused to ELP, repectively; pLI: tetramer motif (GCN4-pLI); pII: trimer motif isoleucine zipper (GCN4-pII); kDa: kilodalton; *: a molecular weight of the trimer; **: a molecular weight of the tetramer.

Recombinant protein	Molecular weight [kDa]	Recombinant protein	Molecular weight [kDa]
HA1	40.3	H5-VLP	62.2
HA2	23.9		
H5	60.8	(H5pII)3	65.2-195.6*
N1	48.4	(pLI-N1)4	52.6-210.4**
HA1-ELP	83.1		
HA2-ELP	64.8		
H5-ELP	101.9	(H5pII-ELP)3	106-318*
N1-ELP	89.5	(pLI-N1-ELP)4	94-376**

The constructs proved by transient expression of the hemagglutinin and neuraminidase variants under control of both the USP and the CaMV 35S promoters were used for stable transformation of tobacco plants (*N. tabacum*). Meanwhile, the infiltrated leaves of *N. benthamiana* that transiently expressed oligomers of hemagglutinins ^{Nb}(H5pII)3, ^{Nb}(H5pII-ELP)3; neuraminidase ^{Nb}(pLI-N1-ELP)4 and ^{Nb}H5-VLP were also used to purify them by either ITC or affinity chromatography (Fig. III-13, 18D, 18C and 15).

III.3. Stable expression of the influenza antigens in transgenic tobacco plants

The expression constructs under control of the CaMV 35S promoter were used to integrate T-DNA into tobacco genome *via* Agrobacterium (section II.2.5). Transformants were generated and selected on medium containing the selecting antibiotics. Young leaves from regenerated transgenic plants (T0) of every construct were used to extract total soluble proteins for expression screening by Western blot using anti-c-myc monoclonal antibody. The number of the regenerated plants and transgenic plants expressing respective proteins were summarized in table III-3.

Table III-3. Transgenic tobacco plants expressing influenza antigens in leaves. Recombinant proteins were detected by Western blot. HA1 and HA2: the subunit 1 and 2 of the hemagglutinin subtype 5; H5 and $(H5pII)_3$: monomer and trimer of the ectodomain hemagglutinin subtype 5, respectively; N1 and $(pLI\text{-}N1)_4$: monomer and tetramer of the ectodomain neuraminidase subtype 1, respectively; HA1-ELP and HA2-ELP: the subunit 1 and 2 of the hemagglutinin subtype 5 fused to ELP, respectively; H5-ELP and $(H5pII\text{-}ELP)_3$: monomer and trimer of the ectodomain hemagglutinin subtype 5 fused to ELP; N1-ELP and $(pLI\text{-}N1\text{-}ELP)_4$: monomer and tetramer of the ectodomain neuraminidase subtype 1 fused to ELP, repectively; pLI: tetramer motif (GCN4-pLI); pII: trimer motif isoleucine zipper (GCN4-pII).

Recombinant protein	Number of regenerated plant	Number of transgene expressing plant
^{Nt}N1	42	23
^{Nt}N1-ELP	37	23
^{Nt}(pLI-N1-ELP)4	115	72
^{Nt}H5	25	18
^{Nt}(H5pII)3	80	53
^{Nt}H5-ELP	55	43
^{Nt}(H5pII-ELP)3	85	70
^{Nt}HA1	36	22
^{Nt}HA1-ELP	88	67
^{Nt}HA2-ELP	102	60

Western blot analyses show that 67 out of 88, 60 out of 102, 43 out of 55, 70 out of 85, 23 out of 37 and 72 out of 115 T0 regenerated plants accumulated the ^{Nt}HA1-ELP, ^{Nt}HA2-ELP, ^{Nt}H5-ELP, ^{Nt}(H5pII-ELP)3, ^{Nt}N1-ELP and ^{Nt}(pLI-N1-ELP)4 in the ER, respectively (Fig. III-8). The immunoblot band intensities in Fig. III-8 demonstrate different expression levels in the single transgenic lines.

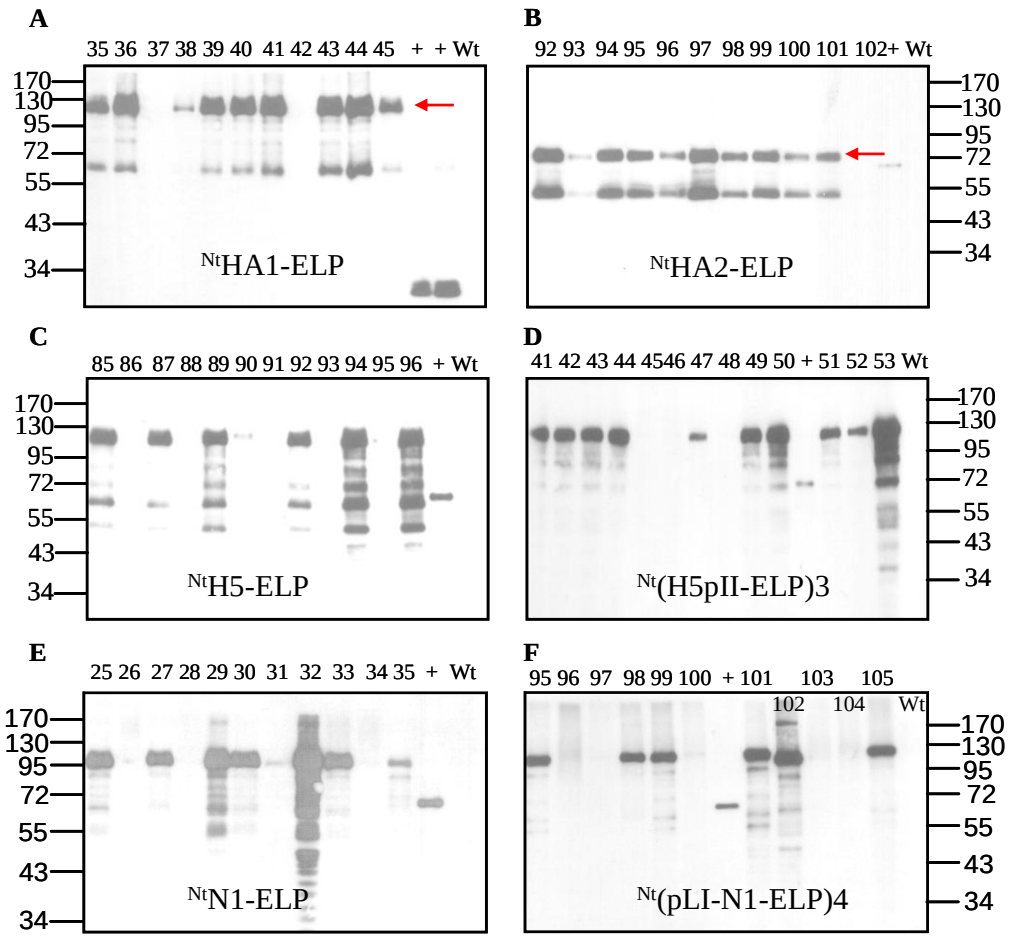

Figure III-8. Expression of ELP fusion hemagglutinins in the transgenic leaves. The transgenic plants express ^{Nt}HA1-ELP (A), ^{Nt}HA2-ELP (B), ^{Nt}H5-ELP (C), ^{Nt}(H5pII-ELP)3, ^{Nt}N1-ELP and ^{Nt}(pLI-N1-ELP)4. 5 µg TSPs were separated by 10% SDS-PAGE, blotted and detected by anti-c-myc monoclonal antibody; +: 1ng ^{Nt}anti-hTNFα-VHH-ELP used as a Western blot standard or 5, 10 ng *E. coli* expressed ScFv (Fig. III-8A); Wt: wild type. The numbers refer to independent primary transgenic plants. Arrows: the position of the target recombinant proteins. Nt: recombinant proteins were expressed stably in *N. tabacum*.

As shown by Western analyses of the transgenic plants expressing ^{Nt}HA1-ELP (Fig. III-8A), ^{Nt}HA2-ELP (Fig. III-8B), immuno-positive bands of smaller proteins were also detectable. That could be due to partial degradations caused by plant proteases.

10 protein extracts from 10 T0 plants that expressed a relatively high level of ^{Nt}H5, ^{Nt}(H5pII)3, ^{Nt}H5-ELP and ^{Nt}(H5pII-ELP)3 were selected to be analysed by Western bot using rabbit anti serum raised against A/Vietnam/1194/2004(H5N1). Western blot analyses show that ^{Nt}H5, ^{Nt}(H5pII)3, ^{Nt}H5-ELP and ^{Nt}(H5pII-ELP)3 were recognized by rabbit anti serum raised against A/Vietnam/1194/2004(H5N1) (Fig. III-9). These results indicate that the plant-derived hemagglutinins (^{Nt}H5, ^{Nt}(H5pII)3, ^{Nt}(H5pII-ELP)3 and ^{Nt}H5-ELP) had an authentic antigenicity. Seeds of these lines were collected and germinated on Kan containing MS

medium for selection of single locus transgene insertion which had a 3:1 segregation (section III.4).

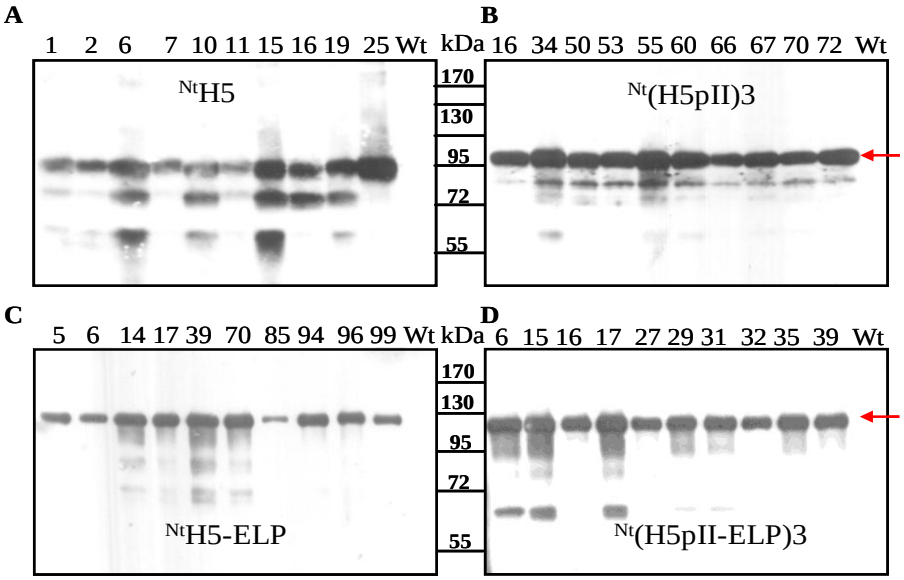

Figure III-9. Expression of hemagglutinins in transgenic plants. The transgenic plants express ^{Nt}H5 (A), ^{Nt}(H5pII)3 (B), ^{Nt}H5-ELP (C) and ^{Nt}(H5pII-ELP)3 (D). 30 µg TSPs were separated by 10% SDS-PAGE, blotted and detected by rabbit anti serum raised against A/Vietnam/1194/2004(H5N1); Wt: wild type. The numbers refer to independent primary transgenic plants. Arrow: the position of the target recombinant proteins. Nt: recombinant proteins were expressed stably in *N. tabacum*.

To monitor an effect of the ELP fusion on influenza antigen accumulation in plant cells, the accumulation levels of ELP fusion antigens and antigens without ELP in leaves were compared by Western blot using anti-c-myc monoclonal antibody under reducing conditions. Although only 3 µg TSP of ELP fusion antigens (^{Nt}N1-ELP, ^{Nt}(pLI-N1-ELP)4, ^{Nt}HA1-ELP, ^{Nt}H5-ELP and ^{Nt}(H5pII-ELP)3) were loaded, their immunoblot signals were comparable with those of 30 µg TSP of influenza antigens without ELP (Fig. III-10). These results indicate that the ELP fusion resulted in a significant increase in the accumulation of influenza hemagglutinin and neuraminidase proteins in leaves. Interestingly, the addition of the tetrameric motif did not influence the expression level of ^{Nt}(pLI-N1-ELP)4 in comparison to ^{Nt}N1-ELP (Fig. III-10A). The fusion to isoleucine zipper (GCN4-pII) resulted in enhancing the expression level of ^{Nt}(H5pII-ELP)3 in comparison to ^{Nt}H5-ELP (Fig. III-10C).

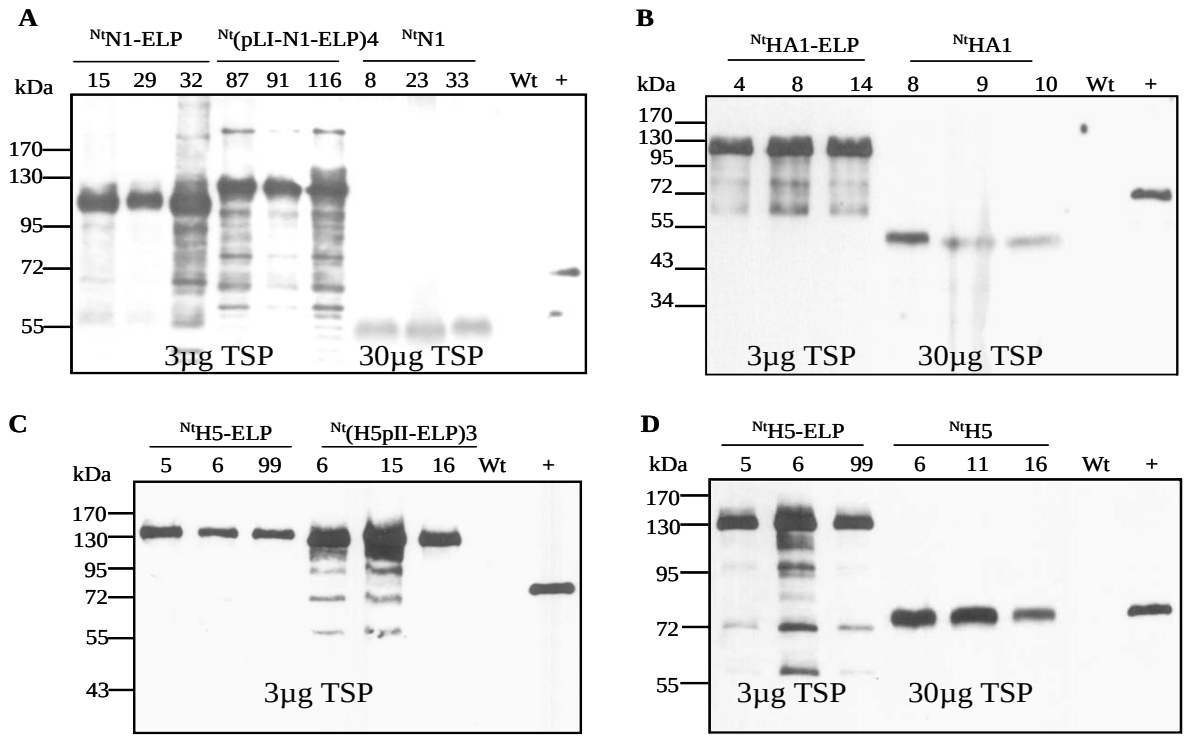

Figure III-10. ELP fusion enhancing expression of influenza antigens in transgenic leaves. Comparing the expression level of ^{Nt}N1-ELP, ^{Nt}(pLI-N1-ELP)4 and ^{Nt}N1 proteins (A), ^{Nt}HA1-ELP and ^{Nt}HA1 (B), ^{Nt}H5-ELP and ^{Nt}(H5pII-ELP)3 (C) ^{Nt}H5-ELP and ^{Nt}H5 (D). 3 µg TSP of ELP fusion proteins (^{Nt}N1-ELP, ^{Nt}(pLI-N1-ELP)4, ^{Nt}HA1-ELP, ^{Nt}H5-ELP and ^{Nt}(H5pII-ELP)3) or 30 µg TSP of no ELP fusion proteins (^{Nt}HA1, ^{Nt}N1 and ^{Nt}H5) were separated by 10% SDS-PAGE, blotted and detected by anti-c-myc monoclonal antibody; +: 1ng ^{Nt}anti-hTNFα-VHH-ELP used as a Western blot standard; Wt: wild type; kDa: kilodalton. The numbers refer to independent primary transgenic plants. Nt: recombinant proteins were expressed stably in *N. tabacum*.

Recombinant proteins which are under control of the seed specific USP promoter were only analysed in ripe seeds. Their total soluble proteins were extracted, separated on SDS-PAGE and analysed by Western blot. As shown in Fig. III-11, prominent bands corresponding to the expected molecular weight of ^{Nt}HA1-ELP, ^{Nt}HA1 (Fig. III-11A), ^{Nt}H5-ELP, ^{Nt}H5 (Fig. III-11B) and ^{Nt}N1-ELP (Fig. III-11C) were detected. Immunoblot signals were not detectable for ^{Nt}N1 proteins (data not shown). Low expression level of this protein was one possible reason. These results implied that ^{Nt}HA1-ELP, ^{Nt}HA1, ^{Nt}H5-ELP, ^{Nt}H5 and ^{Nt}N1-ELP were successfully expressed in transgenic tobacco seeds under control of the seed specific USP promoter. Results in Fig. III-11 also demonstrate that ELP fusion could substantially enhance recombinant fusion proteins in tobacco seeds. The number of the regenerated plants and the transgenic plants that could express recombinant proteins under control of the USP promoter were summarized in table III-4.

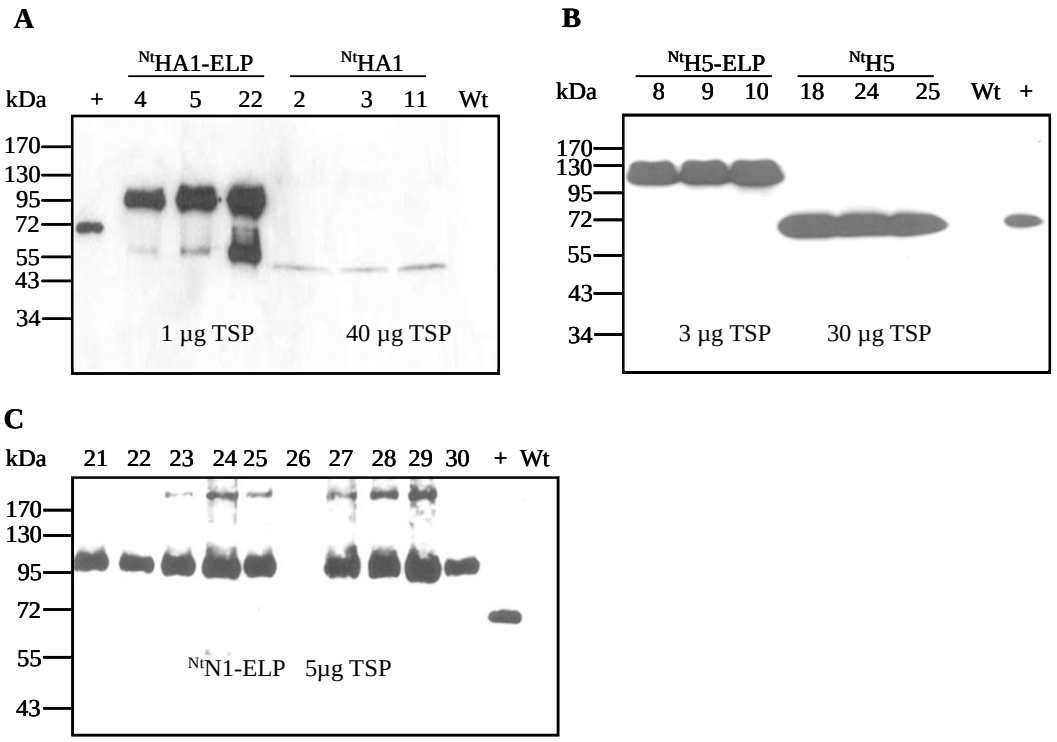

Figure III-11. Expression of ELP fusion hemagglutinins in transgenic seeds. (A) Comparing the expression level of the ^{Nt}HA1-ELP and ^{Nt}HA1 proteins. Amount of TSP for Western blot analysis was 1 and 40 µg, respectively. (B) Comparing the expression level of ^{Nt}H5-ELP and ^{Nt}H5 proteins. 3 µg and 40 µg TSP of the ^{Nt}H5-ELP and ^{Nt}H5 were used for Western blot, respectively. (C) The transgenic seeds expressing ^{Nt}N1-ELP proteins. 5 µg TSP were used for the immunogical analysis. The proteins were separated by 10% SDS-PAGE, blotted and detected by anti-c-myc monoclonal antibody; +: 1ng ^{Nt}anti-hTNFα-VHH-ELP used as a Western blot standard; Wt: wild type; kDa: kilodalton; TSP: total soluble protein. The numbers refer to independent primary transgenic plants. Nt: recombinant proteins were expressed stably in *N. tabacum*.

Table III-4. Transgenic tobacco plants expressing influenza antigens in seeds. Recombinant proteins were sceened by Western blot using anti c-myc monoclonal antibodies. ^{Nt}HA1: the subunit 1 of the hemagglutinin subtype 5; ^{Nt}H5: the ectodomain of the monomer hemagglutinin subtype 5; ^{Nt}N1: the ectodomain of the neuraminidase subtype 1; ^{Nt}HA1-ELP: the subunit 1 of the hemagglutinin subtype 5 fused to ELP; ^{Nt}H5-ELP: the ectodomain of the hemagglutinin subtype 5 fused to ELP; ^{Nt}N1-ELP: the ectodomain of the neuraminidase subtype 1 fused to ELP.

Transgene	Number of regenerated plant	Number of transgene expressing plant
^{Nt}N1	47	0
^{Nt}N1-ELP	45	35
^{Nt}H5	36	25
^{Nt}H5-ELP	91	59
^{Nt}HA1	45	12
^{Nt}HA1-ELP	43	26

III.4. Segregation analysis of the transgenic T1 lines

Segregation analyses were performed to characterize transformed DNA of the *npt II* gene in the first generation. 100 seeds from 10 individual T0 lines that expressed a relatively high level of influenza antigens were germinated on kan containing MS medium. The segregation of resistant and sensitive seedlings was tabulated after five week germination (Fig. III-12) and Chi-square values were used to analyse the significance of an observed deviation from the expected value. The expected segregation is a 3 kanamycin resistant plants (kan^R): 1 kanamycin sensitive plants (kan^S) ratio. Chi-square values are calculated according to the following formula:

$$X^2 = \text{Sum}\frac{(\text{Observed value-Expected value})^2}{(\text{Expected value})}$$

Segregation assays were performed with constructs under control of both the CaMV 35S and USP promoters. Chi-square values of less than 3.841 [the value was obtained from reference tables for biological, agricultural and medical research (Fisher and Yates, 1964)] fit a 3:1 segregation ratio and indicate a single dominant Mendelian locus with 95% of confidence. Plant lines containing single locus integration of the transgenes into the tobacco chromosomes are shown in table III-5. Lines which did not fit a 3:1 segregation were not selected for further analyses and not included in table III-5.

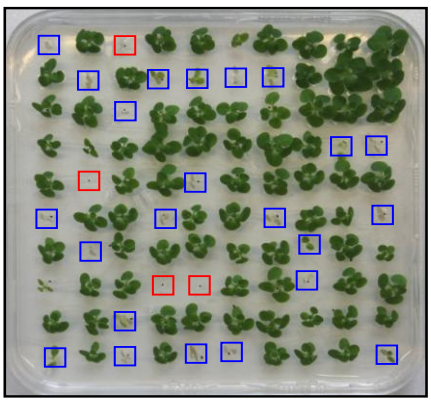

Figure III-12. Segregation analysis of the *npt* II in the progenies of a T0 plant. Segregation analysis of the *npt II* gene in the progenies of a T0 plant (line number 2) expressing H5-ELP under control of the seed specific promoter was performed on selection medium containing kan (section II.2.7). Ungerminated seeds (red) and kan sensitive seedlings (blue) are marked.

10 kanamycin-resistant T1 plantlets that expressed ^{Nt}H5, ^{Nt}H5-ELP, ^{Nt}N1-ELP under control of the CaMV 35S promoter and ^{Nt}H5-ELP, ^{Nt}N1-ELP under control of the seed specific promoter (Table III-5) were selected for transfer to soil and grow in the greenhouse to obtain the next generation. TSPs from leaves of T1 plants expressing ^{Nt}H5, ^{Nt}H5-ELP and ^{Nt}N1-ELP

under control of the CaMV 35S promoter were extracted and analysed by Western lot using anti-c-myc monoclonal antibody. The expression level of recombinant proteins was determined by comparison to known amounts of the standard protein, ^{Nt}anti-hTNFα-VHH-ELP, that containing c-myc tag for Western blot detection. Expression levels of recombinant proteins are shown below.

^{Nt}H5:	0.04%
^{Nt}H5-ELP:	0.5%
^{Nt}(H5pII-ELP)3:	1.0%
^{Nt}N1:	0.004%
^{Nt}N1-ELP:	0.2%
^{Nt}(pLI-N1-ELP)4:	0.2%
^{Nt}HA1-ELP:	0.3%

TSPs from seeds of T1 plants expressing ^{Nt}H5, ^{Nt}H5-ELP and ^{Nt}N1-ELP under control of the seed specific promoter were also extracted and transgenic protein expression was analysed by Western blot using anti-c-myc monoclonal antibody.

Table III-5. Lines with single locus of transgene. Kan^R: kanamycin resistance, kan^S: kanamycin sensitive, χ^2: chi-square value. Chi square values that were less than 3.841 indicate a good fit to a 3 kan resistant: 1 kan susceptible ratio.

Transgene	**T0 Plant line**	**Observed segregation of the *npt II* gene of T1**		**χ^2 value**
		Kan^R	**Kan^S**	
Expression of the antigens under control of the CaMV 35S promoter				
^{Nt}H5	6			
^{Nt}H5-ELP	99	79	17	2.72
^{Nt}N1	12	68	26	0.35
^{Nt}N1-ELP	4	67	24	0.09
^{Nt}HA1-ELP	14	67	29	1.39
^{Nt}(pLI-N1-ELP)4	69	77	23	0.21
Expression of the antigens under control of the USP promoter				
^{Nt}H5	18	68	18	0.76
^{Nt}H5-ELP	2	73	23	0.05
^{Nt}N1-ELP	6	67	25	0.23
^{Nt}HA1	2	65	25	0.37
^{Nt}HA1-ELP	4	71	25	0.05

100 seeds from T1 plants that expressed ^{Nt}H5, ^{Nt}H5-ELP and ^{Nt}N1-ELP under control of the CaMV 35S promoter and ^{Nt}H5-ELP, ^{Nt}N1-ELP under control of the seed specific promoter were germinated on a selective MS medium containing kan to select homozygous

line that have 100% kan resistant plants. One homozygous line for each construct was selected and shown in table III-6.

Table III-6. Selected homozygous lines. Kan^R: kanamycin resistance, kan^S: kanamycin sensitive plants.

Transgene	T0 Parent line	T2 homozygous line	Kan^R	Kan^S	Non-germination
Expression of the antigens under control of the CaMV 35S promoter					
^{Nt}H5	6	6	98	0	2
^{Nt}H5-ELP	99	4	94	0	6
^{Nt}HA1-ELP	14	3	100	0	0
^{Nt}N1-ELP	4	9	96	0	4
Expression of the antigens under control of the seed specific promoter					
^{Nt}H5-ELP	2	10	95	0	5
^{Nt}N1-ELP	6	8	97	0	3

III. 5. Purification of recombinant proteins from transgenic plants

Purified hemagglutinins and neuraminidases were required for antigen characterization, animal immunization and characterization of hemagglutinin specific antibodies by ELISA. Hemagglutinin and neuraminidase proteins expressed in both stable transgenic N. tobaccum plants and transiently transformed *N. benthamiana* plants were purified either by affinity chromatography or ITC. Hemagglutinins without ELP (^{Nt}H5 and ^{Nb}(H5pII)3) were purified from leaf materials using IMAC based on the 6x histidine-tag/Ni-NTA interaction, while the ELP fusion hemagglutinins (^{Nt}H5-ELP and ^{Nb}(H5pII-ELP)3) and neuraminidases (^{Nt}N1-ELP and ^{Nb}(pLI-N1-ELP)4) were enriched by ITC.

III.5.1. Purification of hemagglutinins by immobilized metal affinity chromatography

In order to obtain ^{Nt}H5 and ^{Nb}(H5pII)3 for further experiments, these proteins were expressed as fusion proteins containing a C-terminal 6x histidine tag. ^{Nt}H5 recombinant protein was purified from stable transgenic plant leaves, whereas ^{Nb}(H5pII)3 recombinant protein was enriched from transiently transformed *N. benthamiana* leaves. Samples from each step of the purification procedure were collected and analysed by Coomassie blue stain and Western blot using rabbit anti serum raised against A/Vietnam/1194/2004(H5N1). The results of the ^{Nb}(H5pII)3 and ^{Nt}H5 purification are presented in Fig. III-13 and 14, respectively.

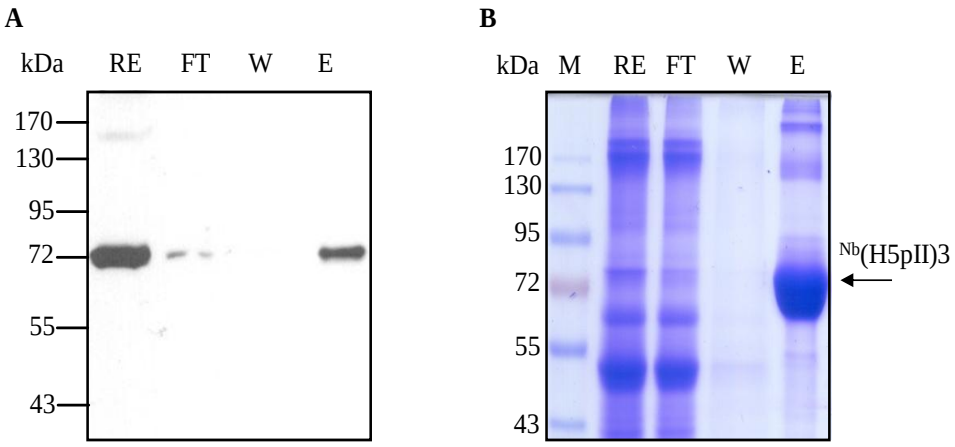

Figure III-13. Purification of ^{Nb}(H5pII)3 by IMAC. 20 µg TSPs of raw extract (RE), flow through (FT) and wash fraction (W) were separated by 10% SDS-PAGE. (A) ^{Nb}(H5pII)3 detection by Western blot. ^{Nb}(H5pII)3 was detected by rabbit anti serum raised against A/Vietnam/1194/2004(H5N1). 50 ng of the purified ^{Nb}(H5pII)3 from elution fraction (E) was separated by 10% SDS-PAGE. (B) ^{Nb}(H5pII)3 detection by Coomassie stain. 3 µg of the purified ^{Nb}(H5pII)3 were loaded on 10% SDS-PAGE. ^{Nb}(H5pII)3: the ectodomain of the hemagglutinin subtype 5 fused with isoleucin zipper GCN4-pII at the C-terminus, M: protein marker, Nb: the recombinant protein was purified from the transiently transformed *N. benthamiana* leaves.

The Western blot analyses (Fig. III-13A and 14A) using rabbit anti serum raised against A/Vietnam/1194/2004(H5N1) showed that ^{Nb}(H5pII)3 and ^{Nt}H5 were efficiently enriched from tobacco plant leaves. These plant-derived hemagglutinins were recognized by rabbit anti serum raised against A/Vietnam/1194/2004(H5N1). This implied that the purified ^{Nb}(H5pII)3 and ^{Nt}H5 had the authentic antigenicity. The purified ^{Nb}(H5pII)3 and ^{Nt}H5 were aslo confirmed by Coomassie blue gels (Fig. III-13B and 14B), respectively. These results indicate that the purified ^{Nt}H5 and ^{Nb}(H5pII)3 were highly pure. The purified ^{Nt}H5 and ^{Nb}(H5pII)3 were further characterized structurally and functionally in section III.6.

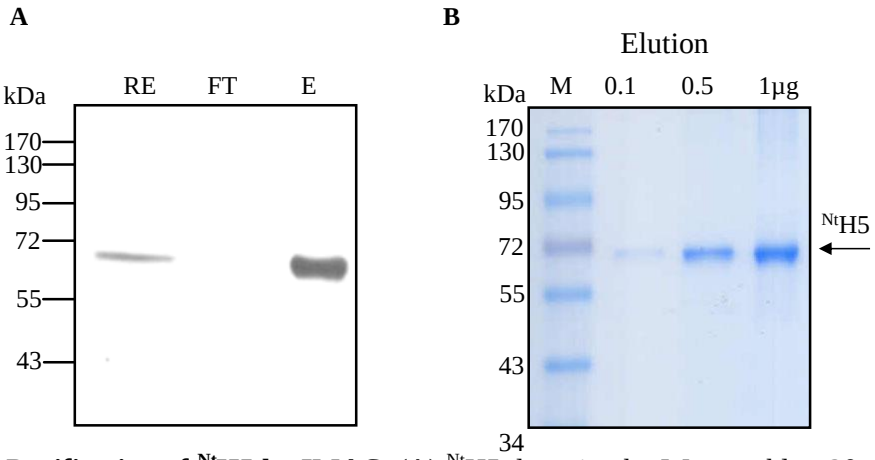

Figure III-14. Purification of ^{Nt}H5 by IMAC. (A) ^{Nt}H5 detection by Western blot. 20 µg TSPs of raw extract (RE), flow through (FT) and 0.1 µg of the purified ^{Nt}H5 from elution fraction (E) were separated by 10% SDS-PAGE. Proteins were then electrotransferred to nitrocellulose membrane and detected by

rabbit anti serum raised against A/Vietnam/1194/2004(H5N1). (B) ^{Nt}H5 detection by Coomassie stain. 0.1, 0.5 and 1µg of the purified ^{Nt}H5 were loaded on SDS-PAGE. ^{Nt}H5: the ectodomain of the hemagglutinin subtype 5, M: protein marker, Nt: the recombinant protein was purified from the transgenic *N. tabacum* leaves. Arrow: the position of the target recombinant protein.

III.5.2. Purification of ^{Nb}H5-VLP by affinity chromatography

Because the homologous inactivated virus strain A/Hatay/2004/(H5N1) was not available, VLPs of this strain were produced in transiently transformed *N. benthamiana* leaves. The hemagglutinin-based VLPs from plants were used for hemagglutination inhibition to characterize chicken and mouse sera (section III.7 and 8). To this purpose, the complete hemagglutinin of the A/Hatay/2004/(H5N1) strain including the TMD, CT domain and legumine B4 signal peptide was expressed transiently in intact leaves of *N. benthamiana*. Fetuin, the major glycoprotein in fetal calf serum, is a sialic acid rich glycoprotein (Baenziger and Fiete, 1979) and used to purify influenza virus hemagglutinin by affinity chromatography. This is based on interaction between the receptor binding site pocket of hemagglutinin and its receptor (sialic acid) (Becht and Rott, 1972; D'Aoust *et al*., 2008).

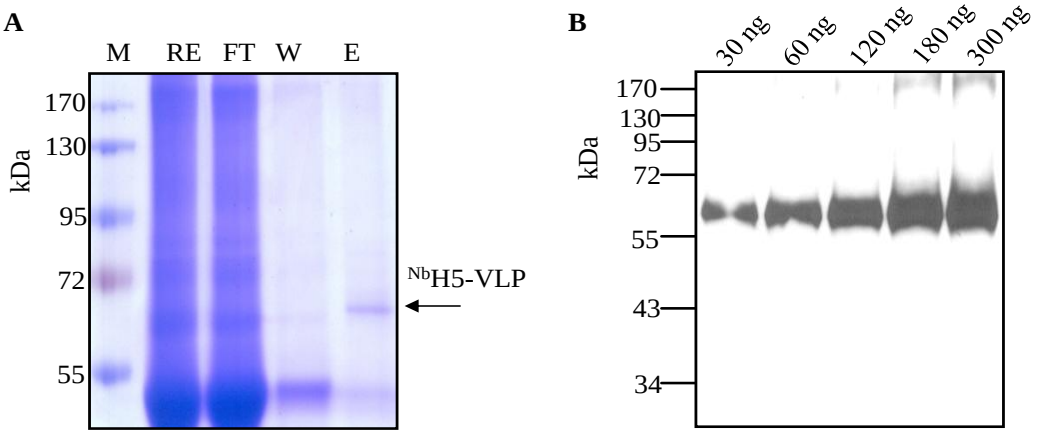

Figure III-15. Purification of ^{Nb}H5-VLP. (A) Protein detection by Coomassie stain. Proteins from raw plant extract (RE), flow through (FT), wash (W) and elution (E) step were separated by 10% SDS-PAGE. M: protein marker. (B) The purified ^{Nb}H5-VLP detection by Western blot. Different concentrations of the purified ^{Nb}H5-VLP from the elution step were separated by 10% SDS-PAGE, electrotransferred to nitrocellulose membrane and detected by rabbit anti serum raised against A/Vietnam/1194/2004(H5N1). M: protein marker. Nb: the recombinant protein was purified from the transiently transformed *N. benthamiana* leaves.

The ^{Nb}H5-VLP proteins were purified following the protocol described by D'Aoust *et al* (2008) using fetuin-conjugated matrix. The purified ^{Nb}H5-VLPs were then analysed on 10% SDS-PAGE, stained by Coomassie blue (Fig. III-15A) and immunodetected by Western blot using rabbit anti serum raised against A/Vietnam/1194/2004(H5N1) (Fig. III-15B). The Coomassie blue and Western blot analyses indicate that ^{Nb}H5-VLPs were successfully purified

to a high purity and authentic antigenicity. Molecular weight of the purified ^{Nb}H5-VLP corresponded to the expected size (about 62.2 kDa). The functionality and structure of the purified ^{Nb}H5-VLP were examined in section III.6.

III.5.3. Purification of the ELP fusion influenza antigens from tobacco leaves

III.5.3.1. Centrifugation-based ITC (cITC)

The ELP fusion strategy provides a simple method for purification of recombinant proteins. This method is based on the inverse phase transition behavior of the ELP tag that is imparted to the ELP fusion proteins. The inverse phase transition of ELP fusion proteins is temperature- and salt-dependent (Meyer and Chilkoti, 1999). An increase in salt concentration (NaCl) and temperature results in formation of insoluble ELP fusion proteins that were separated from the solution by centrifugation at the define temperature. The insoluble ELP fusion proteins were then re-solubilized in a cool and low-ionic buffer. This purification method was described in 1999 and termed as the inverse transition cycling-ITC (Meyer and Chilkoti, 1999). This centrifugation-based ITC (cITC) was applied to purify successfully the antibodies against HIV (Floss *et al*., 2009; Floss *et al*., 2008), mycobacterial antigen (Floss *et al*., 2010a), nanobody (Conrad *et al*., 2011), spider silk proteins (Scheller *et al*., 2004), erythropoietin (Conley *et al*., 2009a) and soluble gp130 (Lin *et al*., 2006) from plant cells.

In this study, six different ELP fusion influenza antigens (^{Nt}HA1-ELP, ^{Nt}HA2-ELP, ^{Nt}H5-ELP, ^{Nb}(H5pII-ELP)3, ^{Nb}(pLI-N1-ELP)4 and ^{Nt}N1-ELP) were purified using cITC. The ^{Nt}HA1-ELP, ^{Nt}HA2-ELP, ^{Nt}H5-ELP and ^{Nt}N1-ELP were purified from stable transgenic plant (*N. tabacum*) leaves, whereas ^{Nb}(H5pII-ELP)3 and ^{Nb}(pLI-N1-ELP)4 were enriched from the transiently transformed *N. benthamiana*.

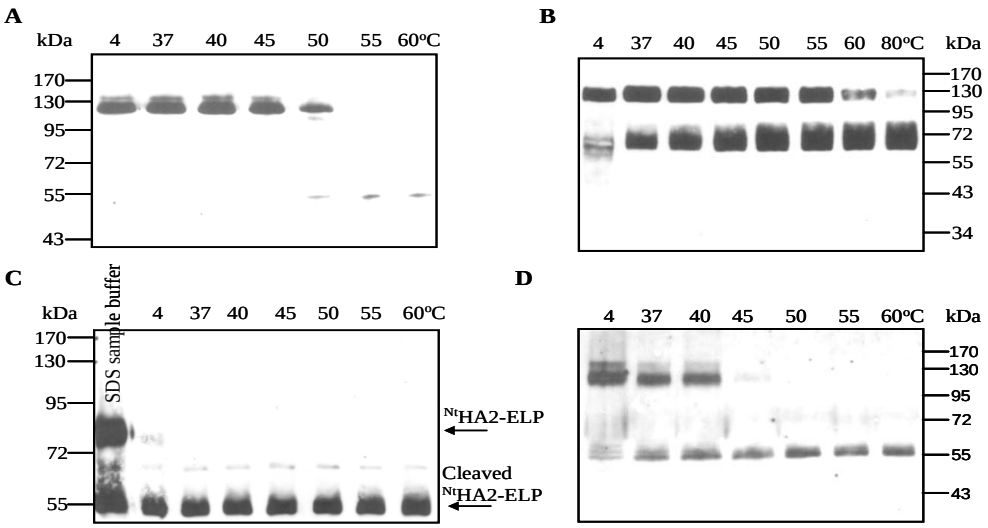

Figure III-16. Examination of temperature stability of ELP fusion proteins. ^{Nt}H5-ELP (A), ^{Nt}HA1-ELP (B), ^{Nt}HA2-ELP (C) and ^{Nt}N1-ELP (D) were extracted in Tris buffer (50 mM, pH 8.0) and incubated for 30 min at different temperatures ranging from 4 to 60 °C (80 °C in case of ^{Nt}HA1-ELP). Protein extracts were centrifuged to remove insoluble materials, separated on 10% SDS-PAGE and detected by Western blot using anti-c-myc monoclonal antibody. ^{Nt}HA2-ELP protein was also solubilized in SDS sample buffer and incubated immediately for 10 min at 95 °C to prove that ^{Nt}HA2-ELP expressed in living plant cells had full length.

Thermal denaturation of ELP fusion proteins (^{Nt}HA1-ELP, ^{Nt}HA2-ELP, ^{Nt}H5-ELP and ^{Nt}N1-ELP) was examined to determine which temperatures cause denaturation of the ELP fusion proteins. Clear plant extracts were incubated for 30 min at different temperatures ranging from 4 to 60 °C or 80 °C for ^{Nt}HA1-ELP and centrifuged to remove insoluble materials. The resulting protein extracts were analysed on 10% SDS-PAGE and immunodetected using anti-c-myc monoclonal antibody. Immunoblot analyses are demonstrated in Fig. III-16 and summarized in table III-7. The Western blot results show that ^{Nt}H5-ELP was partially denaturated at 45 °C and completely denaturated at 55 °C (Fig. III-16A), while ^{Nt}HA1-ELP was partially denaturated at 60 °C and 80 °C (Fig. III-16B). In addition, incubation of protein extracts at high temperatures caused partial cleavage by plant proteases. In case of ^{Nt}HA2-ELP, this protein was extremely sensitive to plant proteases so that intact ^{Nt}HA2-ELP was not observed at all temperatures. The full size of ^{Nt}HA2-ELP was only immunodetected when this protein was solubilized in SDS sample buffer and incubated immediately for 10 min at 95 °C. These results indicate ^{Nt}HA2-ELP was digested immediately by plant proteases after protein extraction. Therefore, this protein was not further characterized. ^{Nt}N1-ELP was partially and completely denaturated at 45 °C and 50 °C, respectively. In summary, the optimal temperatures chosen for purification of the ^{Nt}H5-ELP, ^{Nt}HA1-ELP and ^{Nt}N1-ELP by cITC were 40, 55 and 40 °C, respectively (Table III-7). Purification of ^{Nb}(H5pII-ELP)3 and ^{Nb}(pLI-N1-ELP)4 by cITC were also carried out at 40 °C.

Table III-7. Temperatures for purification of ELP fusion antigens using cITC. ND: not determine, CND: can not determine.

Protein	Denaturing temperature	Temperature for cITC	Note
^{Nt}HA1-ELP		55 °C	Plant protease sensitive
^{Nt}HA2-ELP	CND	CND	Extremely sensitive to plant proteases
^{Nt}H5-ELP	45-55 °C	40 °C	
^{Nb}(H5pII-ELP)3	ND	40 °C	
^{Nt}N1-ELP	45 °C	40 °C	
^{Nb}(pLI-N1-ELP)4	ND	40 °C	

The leaf-derived ^{Nt}HA1-ELP, ^{Nt}H5-ELP, ^{Nb}(H5pII-ELP)3, ^{Nb}(pLI-N1-ELP)4 and ^{Nt}N1-ELP were readily purified by cITC. The supernatants after heat centrifugation (Sc) and proteins captured in the pellet after heat centrifugation and solubilization in water (Pc) were analysed by Western blot (Fig. III-17) and Coomassie blue stain (Fig. III-18).

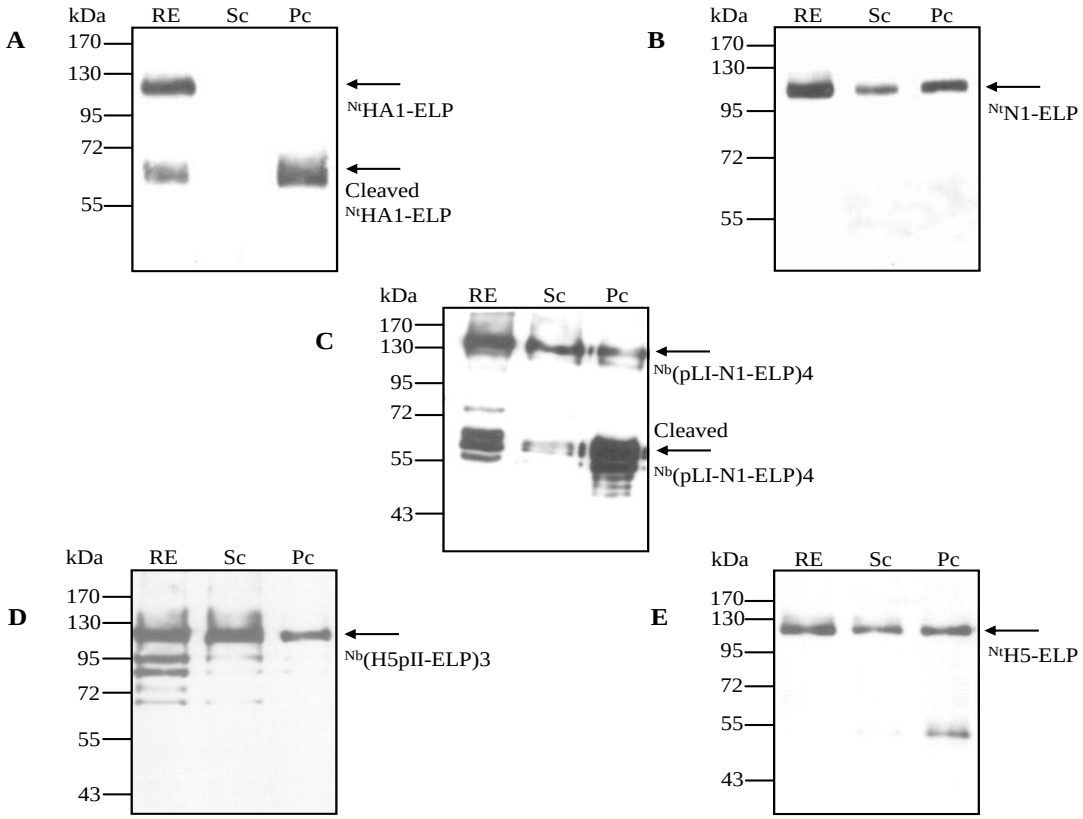

Figure III-17. cITC purification, as demonstrated by Western blot. (A) ^{Nt}HA1-ELP. (B) ^{Nt}N1-ELP. (C) ^{Nb}(pLI-N1-ELP)4. (D) ^{Nb}(H5pII-ELP)3. (E) ^{Nt}H5-ELP. Phase transition was induced by adding NaCl to the plant extracts expressing ^{Nt}HA-ELP, ^{Nt}N1-ELP, ^{Nb}(pLI-N1-ELP)4, ^{Nb}(H5pII-ELP)3 and H5-ELP (final concentration of NaCl was 2 M) and raising the temperature of the plant extracted to 40 ^{o}C or 55 ^{o}C (plant extract expressing the ^{Nt}HA1-ELP) for 15 min. The aggregated ELP fusion proteins were pelletted by the centrifugation for 30 min at 40 ^{o}C and 55 ^{o}C (for ^{Nt}HA1-ELP purification). The pelletted proteins were dissolved in ice-cold water and then insoluble matters were removed by the centrifugation. Proteins from raw extract (RE), supernatant after heat centrifugation (Sc) and proteins captured in the pellet after heat centrifugation and solubilization in water (Pc) were separated by 10% SDS-PAGE, blotted and detected by anti-c-myc monoclonal antibody. Nt and Nb: recombinant proteins were purified from *N. tabacum* and *N. benthamiana*, respectively.

^{Nt}N1-ELP (Pc, Fig. III-17B and 18B) and ^{Nb}(pLI-N1-ELP)4 (Pc, Fig. III-17C and 18C) were detected in the resolubilized precipitates. ^{Nt}N1-ELP (Sc, Fig. III-17B) and ^{Nb}(pLI-N1-ELP)4 (Sc, Fig. III-17C) were also immunodetected in the supernatants after heat centrifugation. Furthermore, the purified ^{Nt}N1-ELP (Pc, Fig. III-18B) and ^{Nb}(pLI-N1-ELP)4 (Pc, Fig. III-18C) were accompanied by other plant proteins resulting a purification efficiency

of 40% and 38% (in terms of protein amount), respectively (Table III-8) and 33.7% (in terms of enzymatic activity of ^{Nt}N1-ELP).

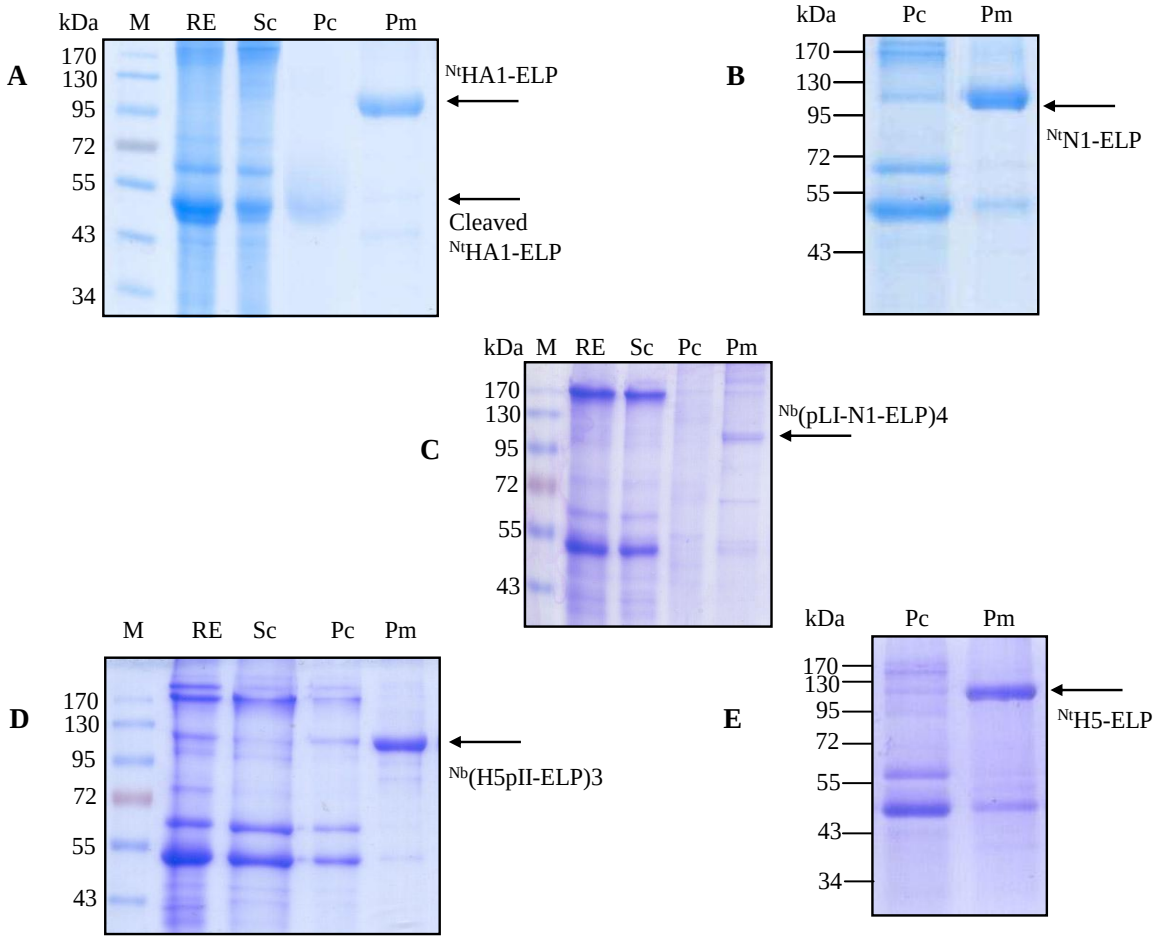

Figure III-18. In gel staining (Coomassie blue) analysis of proteins purified by cITC and mITC. (A) ^{Nt}HA1-ELP. (B) ^{Nt}N1-ELP. (C) ^{Nb}(pLI-N1-ELP)4. (D) ^{Nb}(H5pII-ELP)3. (E) ^{Nt}H5-ELP. The ELP fusion proteins were purified using centrifugation-based ITC or membrane-based ITC. After purification, proteins from RE, Sc and Pc were separated by 10% SDS-PAGE, blotted and detected by anti-c-myc monoclonal antibody. RE: raw extract; Sc: supernatant after heat centrifugation; Pc: proteins captured in the pellet after heat centrifugation and solubilization in ice-cold water; Sm: supernatant after raw extract passed through a 0.2 µm cellulose acetate membrane at room temperature; Pm: proteins eluted from the membrane by ice-cold water. Nt and Nb: recombinant proteins were purified from the transgenic *N. tabacum* and the transiently transformed *N. benthamiana* leaves, respectively.

For the ^{Nt}HA1-ELP protein, the cITC method resulted in the near complete disappearance of the full-length ^{Nt}HA1-ELP; what remained was one cleavage product consisting of the ELP repeats and the c-myc tag (Pc, Fig. III-17A and 18A). The recovery efficicency of this proteins was high (Table III-8). In case of the ^{Nb}(H5pII-ELP)3 and ^{Nt}H5-ELP, the target proteins were immunodetected in the resolubilized precipitates (Pc, Fig. III-17D) and (Pc, Fig. III-17E), respectively or Coomassie blue stain (Pc, Fig. III-18D) and (Pc,

Fig. III-18E), respectively. The ^{Nb}(H5pII-ELP)3 and ^{Nt}H5-ELP were also immunodetected in the supernatants after heat centrifugation (Sc, Fig. III-17D) and (Sc, Fig. III-17E), respectively, resulting a purification efficiency of 67% and 75% (in terms of protein amount), respectively (Table III-8). Again, other plant proteins were contaminated with the ^{Nb}(H5pII-ELP)3 and ^{Nt}H5-ELP in the resolubilized precipitates and detected by Coomassie blue stain (Pc, Fig. III-18D) and (Pc, Fig. III-18E), respectively.

In brief, the purification results of the ELP fusion influenza antigens using cITC show that the purified ELP fusion proteins were impure, plant protease sensitive and revealed low concentration and variably efficient recovery.

III.5.3.2. Membrane-based ITC (mITC)

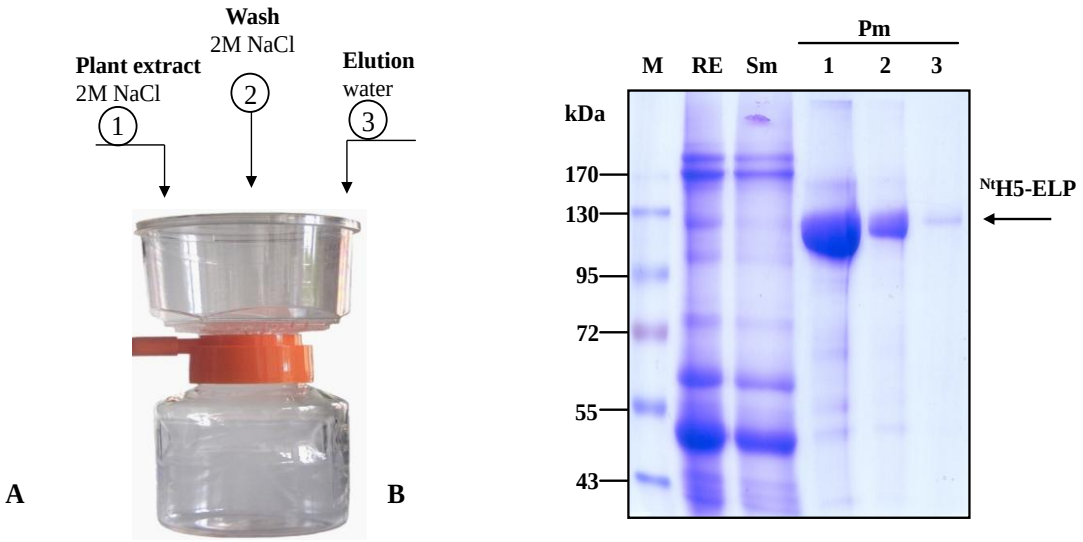

Figure III-19. mITC purification. (A). Diagram of the mITC purification process. Step (1): clear protein plant extract containing 2 M NaCl was raised to room temperature and passed through a 0.2 µm cellulose acetate membrane with a vacuum pump. Step (2): the membrane was washed with warm NaCl 2 M. Step (3): aggregates of ELP fusion proteins were dissolved using ice-cold water and eluted out of the membrane by a vacuum pump. (B) Purification of the ^{Nt}H5-ELP by the mITC. The ^{Nt}H5-ELP was purified from transgenic plant leaves by mITC (Fig. III-19A). 1-3: three elution fractions of Pm obtained by resolubilizing the precipitated ^{Nt}H5-ELP on the membrane surface by the same volumn of ice-cold water and eluted the ^{Nt}H5-ELP from the membrane. RE: raw extract; Sm: supernatant after raw extract passed through a 0.2 µm cellulose acetate membrane at room temperature; Pm: proteins eluted from the membrane by ice-cold water. Nt: recombinant proteins were purified from transgenic *N. tabacum* leaves.

Meyer and co-workers observed that triggering the inverse phase transition results in the formation of micrometer size aggregates of the ELP fusion proteins (Meyer and Chilkoti, 2002). These aggregates of the ELP fusion proteins could be retained on the surface of a membrane. The first proof of principle for purification of ELP fusion proteins using membrane

was assessed and optimized by Ge and co-workers when a thioredoxin ELP fusion protein (Trx-ELP) was purified efficiently from bacterial cell lysate using a syringe and a disposable membrane cartridge connected to an ÄKTA Prime system (Ge *et al.*, 2006). The recovery efficiency of the Trx-ELP using mITC reached to 95%.

In initial attemps to purify ELP fusion proteins from plant extracts using the mITC, a syringe procedure was assessed according to the protocol for purification of ELP fusion proteins from *E. coli* (Ge *et al*., 2006). Unfortunately, that method was not successful since plant extracts containing 2 M NaCl could not pass through a membrane. To overcome the difficulties, the critical improvements were focused on pretreatment of plant extracts by following steps: (1) the clearance of the initial plant extracts by centrifugation at high speed 75,600 *g* instead of 16,000 *g*, (2) the second clearance step after addition of NaCl to 2 M by both centrifugation at high speed 75,600 *g* and then pass through a 0.22 µm polyethersulfone membrane at 4 °C (centrifugation and membrane steps should be performed at 4 °C to be sure that the phase transition is not triggered) and (3) using a vacuum pump to facilitate plant extracts to pass through a 0.2 µm cellulose acetate membrane at room temperature. The mITC for purification of the plant-derived ELP fusion proteins consisting of three steps was adapted and demonstrated in Fig. III-19A. Using this improved mITC, the ^{Nt}H5-ELP was purified from transgenic plant leaves. Fig. III-19B shows the Coomassie blue stained SDS-PAGE gel analysis for the samples collected from purification steps. The result demonstrates that the aggregates of the ^{Nt}H5-ELP were retained selectively on the membrane (Pm, Fig. III-19B), while other plant proteins were passed through a 0.2 µm cellulose acetate membrane (Sm, Fig. III-19B). The aggregates of the ^{Nt}H5-ELP were resolubilized by ice-cold water and eluted out of the membrane with a majority of the target protein in the first, little protein in the second and minimal fusion protein in the third elution fraction (Pm, Fig. III-19B). The Coomassie blue staining gel also shows that the ^{Nt}H5-ELP obtained from mITC was highly pure and concentrated.

The samples that were collected from the purification process were also analysed by Western blot. The results shown in Fig. III-20C indicate that the expected size molecule of the ^{Nt}H5-ELP was successfully purified (Pm, Fig. III-20C). The purification recovery efficiency of this protein was 95% (Table III-8) when amount of the ^{Nt}H5-ELP in supernatant after passing through a 0.2 µm cellulose acetate membrane at room temperature (Sm) and in raw extract (RE) was determined by Western blot using anti-c-myc monoclonal antibody.

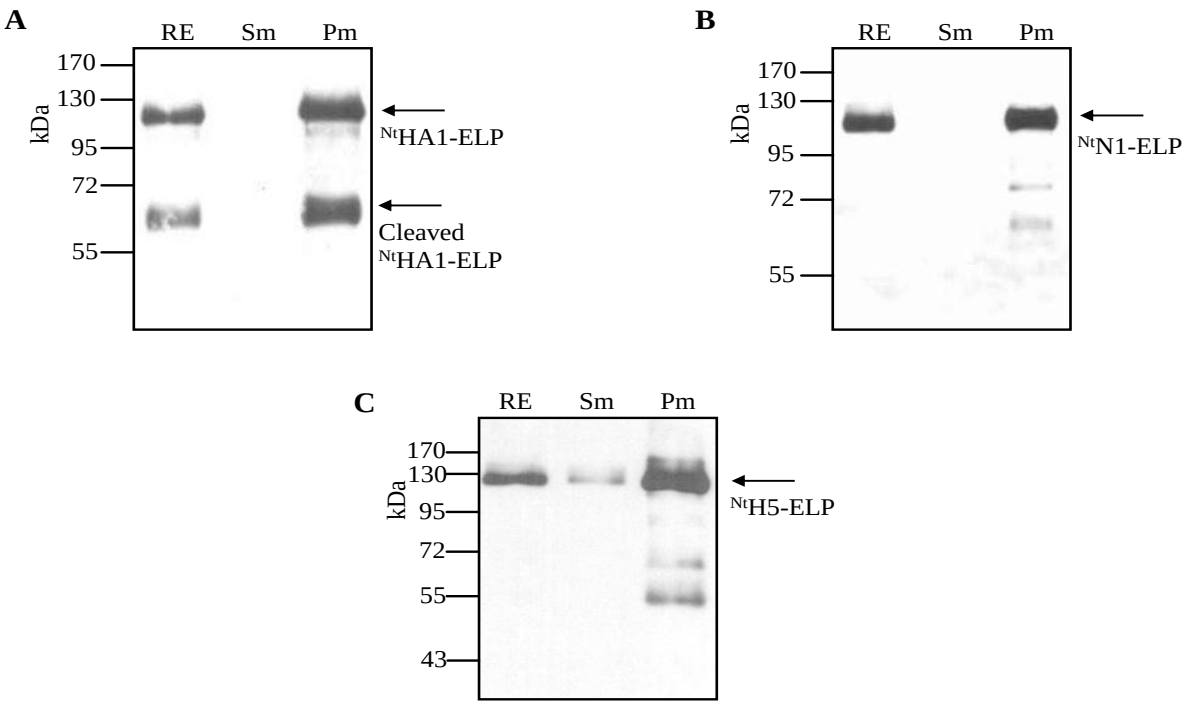

Figure III-20. mITC purification, as demonstrated by Western blot analysis. (A) ^{Nt}HA1-ELP. (B) ^{Nt}N1-ELP. (C) ^{Nt}H5-ELP. RE: raw extract; Sm: supernatant after raw extract passed through a 0.2 µm cellulose acetate membrane at room temperature; Pm: proteins eluted from the membrane by ice-cold water. Nt: recombinant proteins were purified from the transgenic *N. tabacum* leaves.

The ^{Nt}HA1-ELP and ^{Nt}N1-ELP were also enriched from transgenic plant extracts using the same mITC procedure that is used to enrich ^{Nt}H5-ELP. The expected size molecules corresponded to the ^{Nt}HA1-ELP (Pm, Fig. III-20A and 18A) and the ^{Nt}N1-ELP (Pm, Fig. III-20B and 18B) were successfully purified. Losses due to filtration at room temperature and the presence of 2 M NaCl were either insignificant (Sm, Fig. III-20A and 20B) or, at worst and minimal (Sm, Fig. III-20C). Since the resolubilization rate was very high, the mITC purification efficiency was excellent (Table III-8). For NA in particular, the procedure led to a large improvement in the degree of purity achieved (Pm, Fig. III-18B). For the ^{Nt}HA1-ELP, some proteolytic cleavage still occurred, but most of the product remained intact (Pm, Fig. III-20A). The robustness of the mITC method was proven by repeating the procedure four times for each target protein (Fig. III-21).

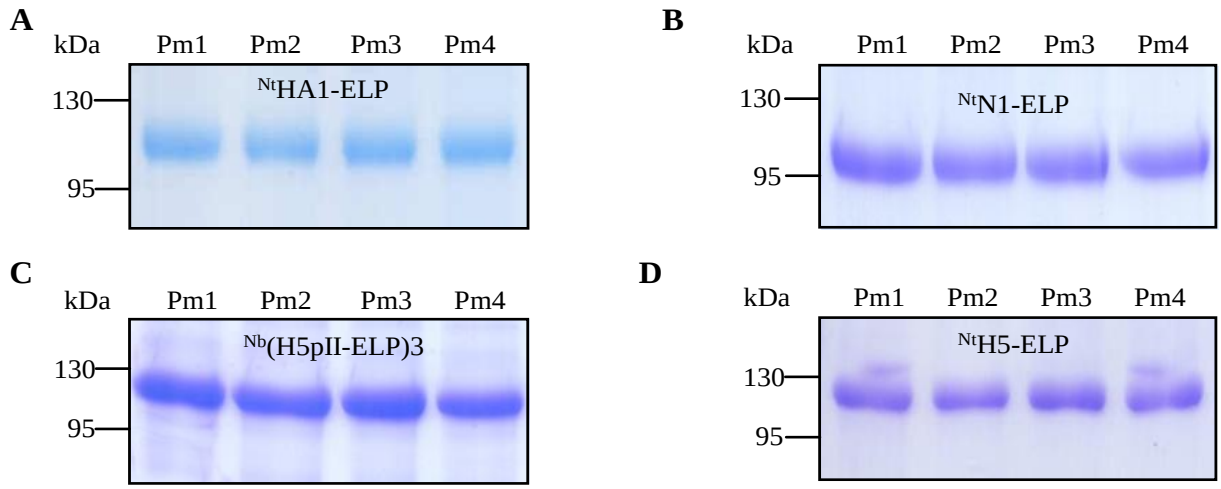

Figure III-21. Robustness of the mITC purification. (A) ^{Nt}HA1-ELP. (B) ^{Nt}N1-ELP. (C) ^{Nb}(H5pII-ELP)3. (D) ^{Nt}H5-ELP. Pm1-Pm4 refer to the four replicated purifications. Protein marker with 95 and 130 kDa shown only. Nt and Nb: recombinant proteins were purified from the transgenic *N. tabacum* and the transiently transformed *N. benthamiana* leaves, respectively.

Table III-8. Efficiency of purification of recombinant target proteins by mITC and cITC. Determined by *amount (Western blot) or **activity. Nt and Nb: recombinant proteins were purified from the transgenic *N. tabacum* and the transiently transformed *N. benthamiana* leaves, respectively; ND: not determine.

Recombinant protein	Recovery (%)		Purity (%)
	mITC	cITC	mITC
^{Nt}HA1-ELP	95*	98*	89
^{Nt}H5-ELP	95*	75*	81
^{Nt}N1-ELP	92.5**-94*	33.7**-40*	81
^{Nb}(H5pII-ELP)3	97*	67*	86
^{Nb}(pLI-N1-ELP)4	97*	38*	ND

As mentioned in section I.1.3.1 and I.1.3.2, the HP H5N1 is a highly potential influenza strain for a next pandemic. In this case, a causative influenza strain could spread very fast and reach to a peak of outbreak between 2-3 months after the first case. The traditional egg-based technology takes 22 weeks to make the first vaccine lots available (Wright, 2008), while transient expression system using *N. benthamiana* leaves offers some benefits in timely fashion and protein quantity. To take advantages of transient expression system, the trimer ELP fusion hemagglutinin ^{Nb}(H5pII-ELP)3 and the tetramer ELP fusion neuraminidase ^{Nb}(pLI-N1-ELP)4 were expressed and purified from the transiently transformed *N. benthamiana* leaves using the mITC procedure. 4 days after agroinfiltration, leaves of the transiently transformed *N. benthamiana* expressing the ^{Nb}(H5pII-ELP)3 and ^{Nb}(pLI-N1-ELP)4 were harvested and

purified using the mITC that was optimized for purification of the ELP fusion proteins from the transgenic plant leaves. However, plant extracts were difficult to pass through a 0.22 µm polyethersulfone membrane at 4 ºC. Therefore, an adapted mITC for purification of ELP fusion proteins from the transiently transformed *N. benthamiana* was optimized (1) by increasing centrifugation times of plant extracts, (2) by descreasing amount of starting leaf material for plant extract (50 g instead of 150 g leaf material) and (3) by passing the plant extract containing 2 M NaCl through a 0.3 µm mixed cellulose ester membrane and then through a 0.22 µm polyethersulfone membrane at 4 ºC. For the ^{Nb}(H5pII-ELP), the expected size molecule was successfully purified from *N. benthamiana* leaves and confirmed by Coomassie blue stain (Pm, Fig. 18D).

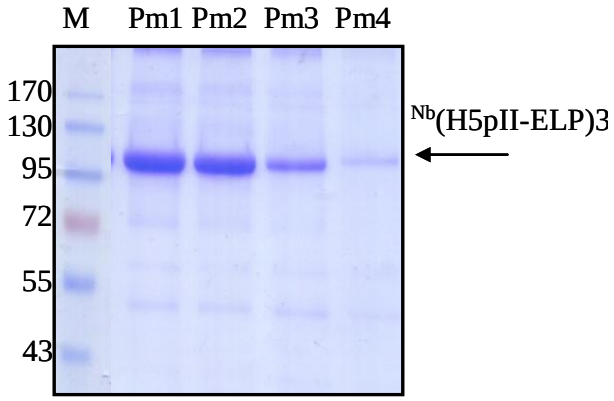

Figure III-22. Purification of ^{Nb}(H5pII-ELP)3 from the transiently transformed *N. benthaminiana.* ^{Nb}(H5pII-ELP)3 was purified with four different rounds of the mITC. The following round of the mITC used the supernatant from the previous round of the mITC. The separation of proteins was performed in SDS-PAGE (10%) under reducing conditions and stained with Coomassie Brilliant Blue. Nb: recombinant proteins were purified from the transiently transformed *N. benthamiana* leaves. M: protein marker, Pm: eluted protein, Pm1 to Pm4: eluted protein from the four different rounds of the mITC.

After the first round of the mITC, the ^{Nb}(H5pII-ELP)3 protein was detected in the Sm sample by Western blot since this protein was incompletely retained on the membrane. The other three rounds of the mITC were carried out. The target proteins of each mITC round were eluted out of a membrane and analysed on a Coomassie blue stained SDS-PAGE gel. The result (Fig. III-22) shows that the ^{Nb}(H5pII-ELP)3 was retained significantly on a 0.2 µm cellulose acetate membrane at room temperature in the first and second round of the mITC, worst and minimal amount of the ^{Nb}(H5pII-ELP)3 was observed in the third and fourth round of the mITC (Fig. III-22). The mITC purification efficiency after the 4th round of the mITC was 97%. For a next purification of the ^{Nb}(H5pII-ELP)3 from the transiently transformed *N. benthamiana* leaves, two rounds of the mITC were required to obtain significant target

proteins. The consistency of the mITC technique has been proven by repeating the procedure 4 times for this protein. The purification technology applied is highly consistent in terms of purity of the product and the efficiency of the process (Fig. III-21C). The ^{Nb}(H5pII-ELP)3 was also purified from *N. benthamiana* leaves by the cITC but its purity and efficiency was much lower than those obtained using the mITC (Pc, Fig. III-18D and Table III-8).

The ^{Nb}(pLI-N1-ELP)4 were also purified from transiently transformed *N. benthamiana* leaves using the same mITC and cITC procedures for purification of the ^{Nb}(H5pII-ELP)3. The expected size molecule corresponded to the ^{Nb}(pLI-N1-ELP)4 was successfully enriched, immunodetected (Pc, Fig. III-17C) and Coomassie blue stained (Pc and Pm, Fig. III-18C).

In summary, the membrane-based ITC method was successfully optimized to purify ELPylated influenza antigens from tobacco leaves. This method is simple, highly efficient, scalable and less time-consuming, especially to avoid degradation of recombinant influenza antigens caused by plant proteases.

III.6. Structural and functional characterization of the purified antigens

III.6.1. Structural characterization

After purification of the ^{Nt}H5, ^{Nt}H5-ELP, ^{Nb}(H5pII)3, ^{Nb}(H5pII-ELP)3, ^{Nt}N1-ELP and ^{Nb}(pLI-N1-ELP)4 proteins from tobacco plant leaves, the oligomeric states of these proteins were determined by cross-linking reaction using BS3 chemical, a water-soluble and homo-bifunctional cross-linker which reacts with primary amines of proteins to form stable amide bonds. When oligomeric proteins were exposed to BS3, cross-links between each subunit of the multimeric proteins are formed. This is a direct evidence for their close proximity. Following cross-linking, reaction products were separated on gradient 4-10% SDS-PAGE under reducing conditions, blotted and immunodetected using anti-c-myc monoclonal antibody. Immunoblot results are demonstrated in Fig. III-23. For the ^{Nt}H5, the immunoblot detection revealed a major band with an apparent molecular weight of 72 kDa and a minor band with a molecular weight of 140 kDa (Fig. III-23A). These bands corresponded to a monomer and dimer of hemagglutinin, respectively. The ^{Nb}(H5pII)3 was appreared as a band with molecular weight of 210 kDa (Fig. III-23A) after the ^{Nb}(H5pII)3 was cross-linked by BS3. These results indicate that the ^{Nt}H5 was a mixture of dimeric proteins and monomeric proteins. The proportion of the monomeric hemagglutinin was markedly higher. ^{Nb}(H5pII)3 was an exclusively trimeric protein.

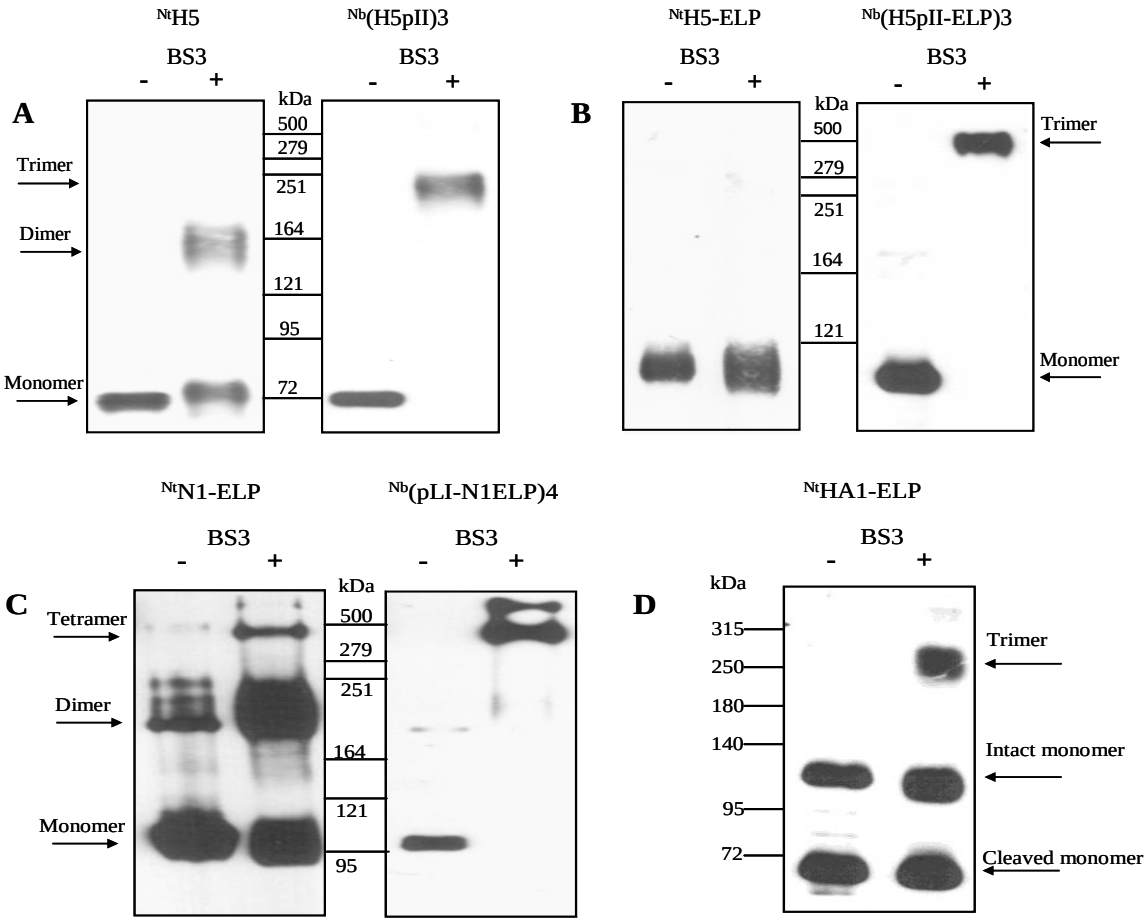

Figure III-23. Detection of oligomeric states of hemagglutinins and neuraminidases by a cross-linking reaction. Chemical cross-linking reactions were performed by mixing bis(sulfosuccinimidyl) suberate (BS3) cross-linker and the purified proteins. The resulting products were separated by 4-10% gradient SDS-PAGE under reducing conditions, blotted and detected by anti-c-myc monoclonal anbody. (-) and (+): indicate a cross-linker with 0 mM and 5 mM final concentration, respectively. (A) ^{Nt}H5 and ^{Nb}(H5pII)3. (B) ^{Nt}H5-ELP and ^{Nb}(H5pII-ELP)3. (C) ^{Nt}N1-ELP and ^{Nb}(pLI-N1ELP)4. (D) ^{Nt}HA1-ELP.

When the ^{Nt}H5-ELP and ^{Nb}(H5pII-ELP)3 were cross-linked individually by BS3 and analysed on the 4-10% gradient SDS-PAGE, bands with molecular weight of 100 kDa and 300 kDa were immunodetected, respectively. These molecular weights correspond to monomer and trimer of the ^{Nt}H5-ELP and ^{Nb}(H5pII-ELP)3, respectively, while proteins that were not exposed to BS3 always appeared as a band with molecular weight of 100 kDa (Fig. III-23B). These results imply that the ^{Nt}H5-ELP was a monomeric protein, while the ^{Nb}(H5pII-ELP)3 was a trimeric protein. The result of cross-linking experiment with ^{Nt}HA1-ELP protein showed that the protein was a mixture of monomeric and trimeric proteins since two immunogical bands that correspond to 110 kDa and 250 kDa were detected. The proportion of trimeric proteins was remarkably lower than that of monomeric proteins (Fig. III-23D).

Cross-linking experiments were also carried out with the ^{Nt}N1-ELP and ^{Nb}(pLI-N1-ELP)4. Fig. III-23C shows that the ^{Nt}N1-ELP existed as a mixture of monomeric (~90 kDa), dimeric (~ 180 kDa) and tetrameric proteins (~360 kDa). The proportions of monomeric and dimeric proteins were high while the proportion of the tetrameric ^{Nt}N1-ELP was lower. Interestingly, the modification of the ELP fusion neuraminidase at N-terminal neuramidase with the GCN4-pLI tetramerization motif could stabilize the oligomeric structure of the ^{Nb}(pLI-N1-ELP)4 protein (Fig. III-23C). The cross-linked ^{Nb}(pLI-N1-ELP)4 appeared as a band of approximately 400 kDa. This result implies that ^{Nb}(pLI-N1-ELP)4 was an exclusive tetramer.

The blue native PAGE (BN-PAGE) is able to determine oligomeric states of proteins under native conditions (Wittig *et al*., 2006). Using this approach, the purified hemagglutinin proteins (^{Nt}H5, ^{Nb}(H5pII)3, ^{Nt}H5-ELP and ^{Nb}(H5pII-ELP)3) were separated on 3.5-10% gradient BN-PAGE, blotted and immunodetected using anti-c-myc monoclonal antibody (section II.2.6.1 and II.2.6.2). When GCN4-pII trimeric motif (isoleucine zipper) was fused to hemagglutinin at the C-terminus, the resulting ^{Nb}(H5pII)3 was an oligomeric protein (lane 4, Fig. III-24). Hemagglutinin without the GCN4-pII trimeric motif (^{Nt}H5) was a clearly monomeric protein with faster movement on BN-PAGE in comparison to ^{Nb}(H5pII)3 (lane 3, Fig. III-24). Lane 1 and 2 in Fig. III-24 also show that mobilities of the ^{Nt}H5-ELP and ^{Nb}(H5pII-ELP)3 on BN-PAGE were clearly different. This result reveals that molecular weight of the ^{Nb}(H5pII-ELP)3 was bigger than that of the ^{Nt}H5-ELP. The oligomeric states of all proteins are shown in table III-9.

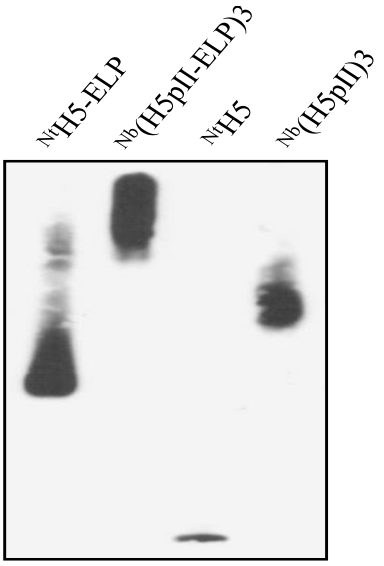

Figure III-24. Oligomeric states of hemagglutinins on blue native PAGE. The purified hemagglutinin proteins were separated by 3.5-10% gradient blue native PAGE under native conditions, blotted and detected by anti-c-myc monoclonal antibody.

Table III-9. Oligomeric states of plant-derived hemagglutinin and neuraminidase proteins. Oligomeric states of plant-derived hemagglutinin and neuraminidase proteins were determined and confirmed by BN-PAGE and cross-linking experiments, "+": indicates an oligomeric state of protein with a lower proportion, "+++": indicates an oligomeric state of protein with a higher proportion.

Protein	Monomer	Dimer	Trimer	Tetramer
^{Nt}HA1-ELP	+++		+	
^{Nt}H5-ELP	+++			
^{Nt}N1-ELP	+++	+++		+
^{Nt}H5	+++	+		
^{Nb}(H5pII)3			+++	
^{Nb}(H5pII-ELP)3			+++	
^{Nb}(pLI-N1-ELP)4				+++

III.6.2. Functional characterization

Purification procedures need to be optimized not only with respect to their recovery efficiency, but also to maintain biological activity. The ELPylated ^{Nt}N1-ELP protein expressed in transgenic plants and purified by mITC was highly soluble even at relatively high concentrations (Fig. III-18B and Fig. III-21B). The enzymatic activity of the ^{Nt}N1-ELP, assessed on the basis of its ability to cleave 2'-(4-methylumbelliferyl)-α-D-N-acetylneuraminic acid, was over four fold that of the cITC purified equivalent (Table III-10). The enzymatic activity of the ^{Nb}(pLI-N1-ELP)4 was also determined using the same assay. Surprisingly, neuraminidase activity of the ^{Nb}(pLI-N1-ELP)4 was not observed although this protein was purified successfully from transiently transformed *N. benthamiana* by the mITC (Pm, Fig. III-18C) and IMAC (Fig. VII-2A). However, the ^{Nt}(pLI-N1-ELP)4 in raw extracts from the transgenic plants showed the enzymatic activities at different levels (Fig. III-25). When the ^{Nt}(pLI-N1-ELP)4 was purified from the transgenic plants by IMAC (Fig. VII-2B), the purified neuraminidase exhibited an excellent specific activity of 314 U/mg. This specific activity of the ^{Nt}(pLI-N1-ELP)4 was over 9-fold that of the ^{Nt}N1-ELP which was purified by mITC. This big difference could come from the different amount of tetrameric neuraminidase. The ^{Nb}(pLI-N1-ELP)4 existed as the executively tetramer, while the proportion of the tetrameric ^{Nt}N1-ELP was remarkably lower (Fig. III-23C).

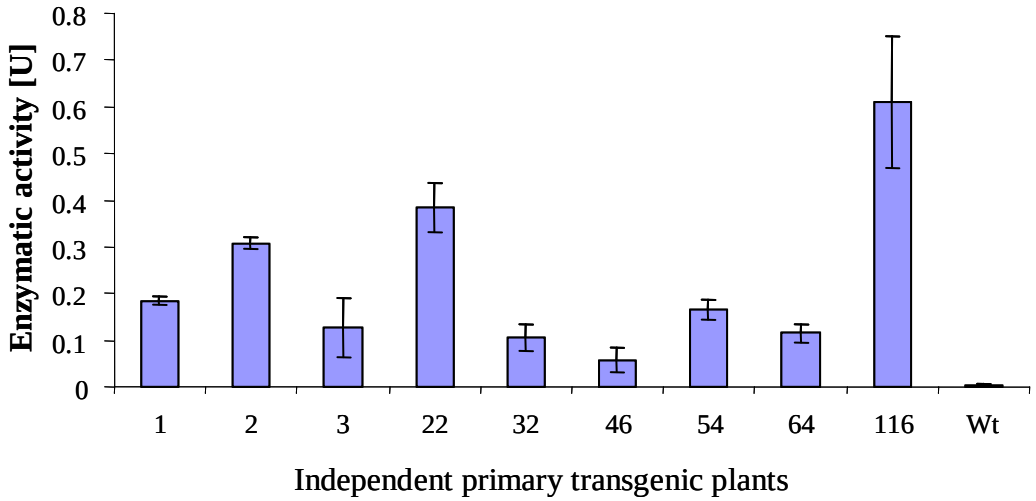

Figure III-25. The enzymatic activity of the ^{Nt}(pLI-N1-ELP)4 from nine stable transformants. Wt: wild type tobacco. Proteins were extracted in buffer containing 20 mM Tris, 4 mM $CaCl_2$, pH 6.5.

Table III-10. The enzymatic activity of the recombinant neuraminidase proteins. The ^{Nt}N1-ELP and ^{Nt}(pLI-N1-ELP)4 proteins were purified from transgenic plants; the ^{Nb}(pLI-N1-ELP)4 was purified from the transiently transformed *N. benthamiana*. The ^{Nt}N1-ELP activity was determined by fluorescence assay before and after purification by mITC or cITC. Data were calculated for 1 kg of leaf material. IMAC: immobilized metal affinity chromatography, mITC: membrane-based inverse transition cycling, cITC: centrifugation-based inverse transition cycling. Nt and Nb: recombinant proteins were purified from the transgenic *N. tabacum* and the transiently transformed *N. benthamiana* leaves, respectively.

Status of neuraminidase	Total protein (mg)	Total activity	Recovery rate (%)	Specific activity (U/mg)
Raw extract	3521.9	736.5	100	0.21
Purified ^{Nt}N1-ELP by mITC	20.3	681.1	92.5	33.6
Purified ^{Nt}N1-ELP by cITC	31.2	248.5	33.7	7.96
Purified ^{Nb}(pLI-N1-ELP)4 by mITC and IMAC				No enzymatic activity
Purified ^{Nt}(pLI-N1-ELP)4 by IMAC				314

The biological activity of the purified hemagglutinin proteins was assessed using hemaglutination assay (section II.2.11.2) based on their ability to bind to receptors which are present on the surface of red blood cells. This binding causes the formation of a lattice termed as hemagglutination. Hemagglutination assay results are shown in Fig. III-26 and Fig. III-27. These results demonstrate that the ^{Nb}(H5pII-ELP)3, ^{Nb}H5-VLP and inactivated virus (positive control) were able to agglutinate chicken red blood cells, while the ^{Nt}H5-ELP and PBS (negative control) were not. The hemagglutination assay of the ^{Nb}(H5pII)3, ^{Nt}H5 and ^{Nt}HA1-

ELP was also determined using the same procedure. The results of the assay were summarized in table III-11.

In summary, the functional and structural data demonstrate that the functionalities of hemagglutinins and neuraminidases were only observed with trimeric and tetrameric hemagglutinin and neuraminidase, respectively. This indicated that the modification of hemagglutinin at the C-terminus with GCN4-pII and neuraminidase at the N-terminus with GCN4-pLI could help these proteins to obtain the native structures and functionalities. The enzymatic activity was observed only with the ^{Nt}(pLI-N1-ELP)4 purified from transgenic plants but not with the ^{Nb}(pLI-N1-ELP)4 purified from transiently transformed *N. benthamiana*.

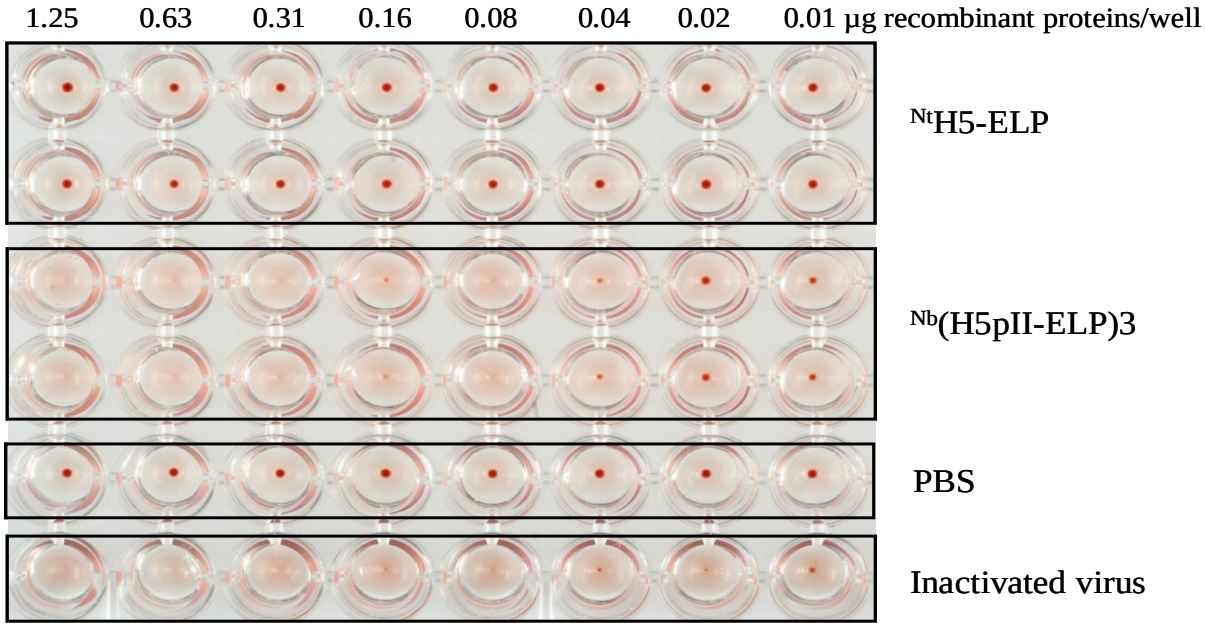

Figure III-26. Hemagglutination assay of the purified ^{Nt}H5-ELP and ^{Nb}(H5pII-ELP)3. Two-fold serial dilution of the purified antigens and inactivated virus were applied across the entire row. The equal volume of PBS (25 µl) was added before 25 µl of 1% chicken red blood cells were added. Hemagglutination result was read after the plate was incubated 40 min at 25 °C. PBS buffer was used as a negative control, while inactivated virus strain rg A/swan/Germany/R65/2006(H5N1) was used as a positive control. The ^{Nt}H5-ELP and ^{Nb}(H5pII-ELP)3 were purified from transgenic plants and transiently transformed *N. benthamiana*, respectively.

Table III-11. Functional and structural assessment of the hemagglutinin proteins. Nt and Nb indicate that recombinant proteins were purified from the transgenic *N. tabacum* and the transiently transformed *N. benthamiana* leaves, respectively.

Antigen	Hemagglutination assay	Oligomeric state
^{Nt}H5-ELP	Negative	Monomer
^{Nb}(H5pII-ELP)3	Positive	Trimer
^{Nt}H5	Negative	Monomer
^{Nb}(H5pII)3	Positive	Trimer
^{Nt}HA1-ELP	Negative	Major monomer and minor trimer
^{Nb}H5-VLP	Positive	Virus-like particle

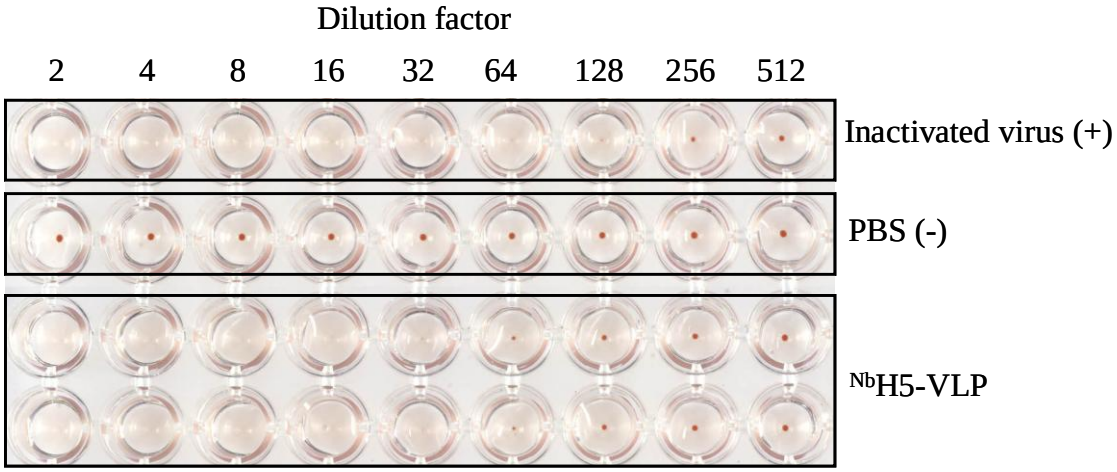

Figure III-27. Hemagglutination assay of the purified ^{Nb}H5-VLP. Two-fold serial dilution of the purified ^{Nb}H5-VLP and inactivated virus were applied across the entire row. The equal volume of PBS (25 µl) was added and then 25 µl of 1% chicken red blood cells were added. Hemagglutination result was read after the plate was incubated 40 min at 25 °C. PBS buffer was used as a negative control while inactivated virus strain rg A/swan/Germany/R65/2006(H5N1) was used as a positive control. The ^{Nb}H5-VLPs were purified from transiently transformed *N. benthamiana* using fetuin-linked agarose.

After the ^{Nb}H5-VLP was purified successfully by affinity chromatography using fetuin-linked agarose (Fig. III-15), the size and morphology of the ^{Nb}H5-VLP were further investigated by electron microscopy. This examination shows that the ^{Nb}H5-VLP formed typically spherical particles with 60-100 nm in size (Fig. III-28). This morphology and size of the ^{Nb}H5-VLP are comparable with those of influenza virus.

Taking functional and structural characterization of the ^{Nb}H5-VLP into consideration, the complete hemagglutinin with transmembrane and cytoplasmic tail domains could form

virus-like particles from plant cells. These particles show similar properties to influenza virions. ^{Nb}H5-VLP was used for hemagglutination inhibition assay in section III-7 and III-8.

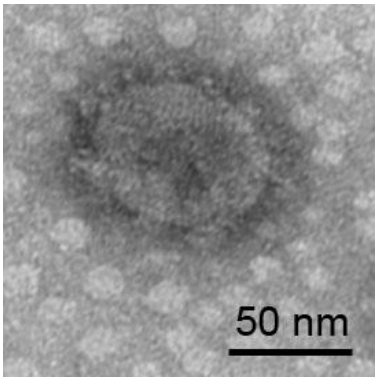

Figure III-28. Electron micrograph of negatively stained influenza ^{Nb}H5-VLP. The ^{Nb}H5-VLP was purified from *N. benthamiana* by affinity chromatography using a fetuin-conjugated agarose. The bar represents 50 nm.

After the recombinant proteins were purified either by IMAC or the mITC, their concentrations were determined by bradford method using BSA protein as a standard protein. The same amount protein (2 µg/lane or 100 ng/lane) was analysed on 10% SDS-PAGE, detected by Coomassie blue stain (Fig. III-29A) or Western blot using rabbit anti serum raised against A/Vietnam/1194/2004(H5N1) (Fig. III-29B). The results indicate that the protein concentrations of the recombinant proteins were comparable. These proteins were used for mouse immunization in section III.8.

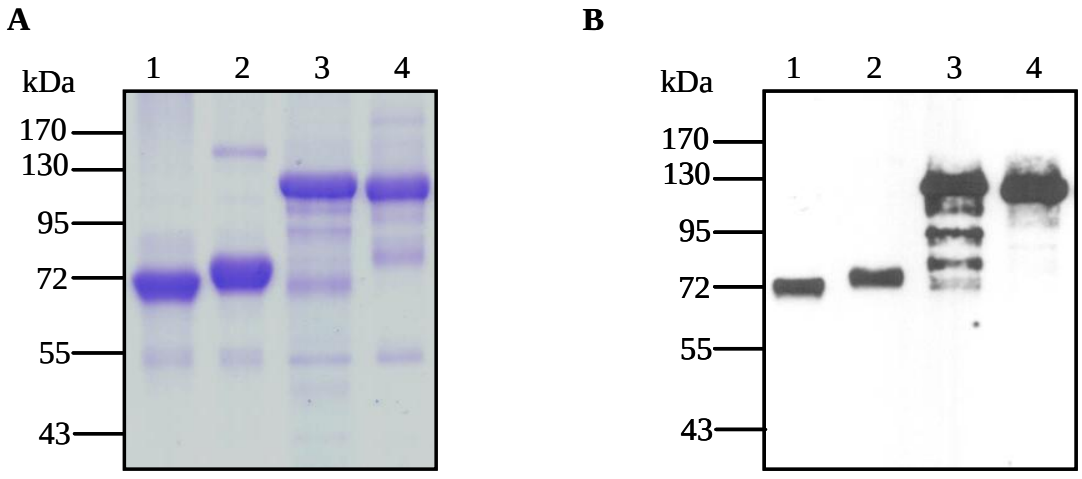

Figure III-29. The purified hemagglutinin proteins for animal immunization. (A) Coomassie blue stain. (B) Western blot detection. Hemagglutinin proteins were separated by 10% SDS-PAGE, electrotransferred to nitrocellulose membrane and detected by rabbit anti serum raised against A/Vietnam/1194/2004(H5N1). Lane 1: ^{Nt}H5, lane 2: ^{Nb}(H5pII)3, lane 3: ^{Nt}H5-ELP, lane 4: ^{Nb}(H5pII-ELP)3.

III.7. Immunogenicity in chickens

In the first animal experiment, the immunogenicity of the monomer hemagglutinins (^{Nt}HA1-ELP and ^{Nt}H5-ELP) was evaluated in chickens. Chickens were vaccinated with a single dose of 10 and 100 µg of Freund adjuvanted ^{Nt}HA1-ELP and ^{Nt}H5-ELP, 18 days after the first immunization (Fig. III-30A) (section II.2.13), chicken sera were collected for detection of the ^{Nt}HA1-ELP and ^{Nt}H5-ELP-specific antibody responses by ELISA.

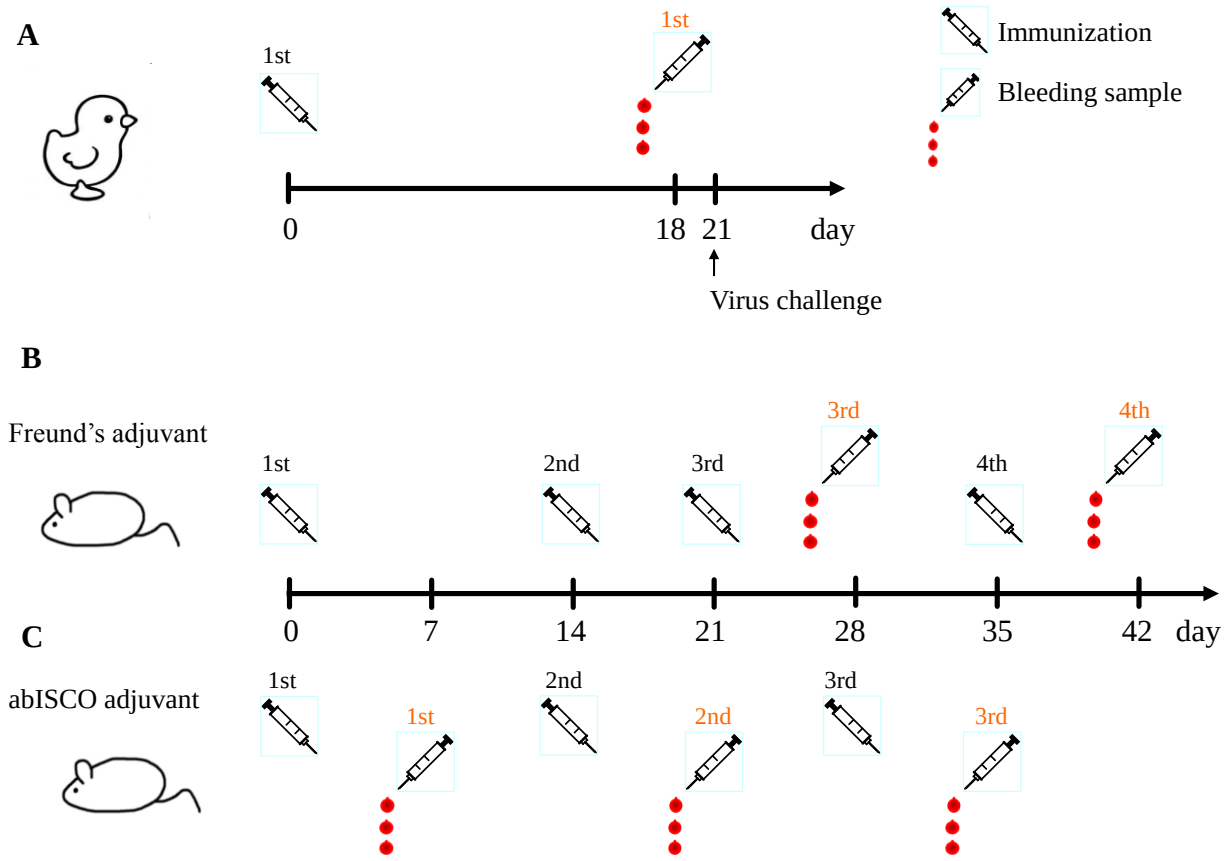

Figure III-30. Outline of the immunization scheme. (A) The immunization study in chickens. Chickens were immunized a single dose of 10 µg and 100 µg of Freund adjuvanted ^{Nt}HA1-ELP and ^{Nt}H5-ELP. 18 days after the first immunization, blood samples were collected. (B) The immunization study in mice with Freund's adjuvant. Mice were immunized four times wih 10 µg and 50 µg of Freund adjuvanted ^{Nt}H5, ^{Nb}(H5pII)3, ^{Nt}H5-ELP and ^{Nb}(H5pII-ELP)3. One week after the 3rd and 4th immunization, blood samples were collected for HI and ELISA tests. (C) The immunization study in mice with abISCO adjuvant. Mice were immunized three times with 10 µg of abISCO adjuvanted ^{Nb}(H5pII)3 and ^{Nb}(H5pII-ELP)3. One week after each immunization, blood samples were collected for HI and ELISA tests. 1st, 2nd, 3rd and 4th indicate bleeding samples were collected after 1st, 2nd, 3rd and 4th immunization, respectively.

The ELISA results reveal that antibody responses could be detected in all animals that were vaccinated with ^{Nt}HA1-ELP and ^{Nt}H5-ELP. The immune responses were dose-dependent (Fig. III-31). Chicken sera were also used to determine HI titers by the HI assay. The HI assay

is a serological standard assay to measure neutralizing antibodies that bind to the hemagglutinin receptor binding pocket. This binding blocks viruses to attach to sialic acid receptor resulting in aggregation of chicken red blood cells. Because the homologous inactivated virus of A/Hatay/2004/(H5N1) strain was not available, the heterologous inactivated rg A/swan/Germany/R65/2006(H5N1) virus and the homologous virus-like particles (^{Nb}H5-VLPs) were used in HI assays. The sera from all groups had no detectable HI activity.

Chickens vaccinated with ^{Nt}HA1-ELP and ^{Nt}H5-ELP were challenged with HPAI A/duck/Vietnam/TG24-01/05(H5N1) strain 21 days after the first immunization. Chickens from all groups died two days after virus challenge. Only one animal that received 100 µg of ^{Nt}HA1-ELP died three days after virus challenge. Taken together, these results indicate that neutralizing antibodies were not induced by the single dose of ^{Nt}H5-ELP and ^{Nt}HA1-ELP hemagglutinins. Chickens that were vaccinated with the single dose of ^{Nt}H5-ELP and ^{Nt}HA1-ELP were not protected from lethal virus.

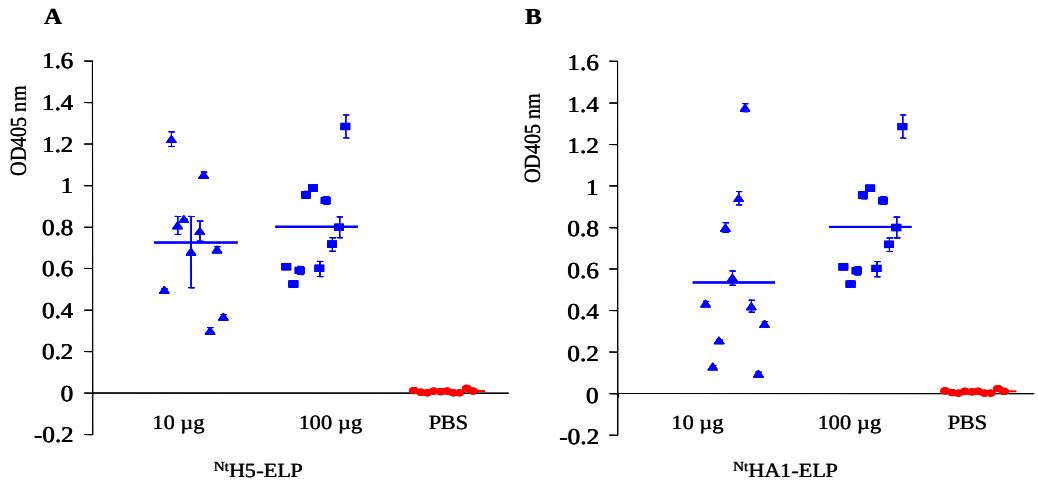

Figure III-31. Chicken antibody responses, as determined by ELISAs. Four groups of 10 chickens were immunized intramuscularly with a single dose of 10 and 100 µg either ^{Nt}H5-ELP or ^{Nt}HA1-ELP. One group was mock-treated with PBS as a negative control group. (A) Chicken sera against ^{Nt}H5-ELP. (B) Chicken sera against ^{Nt}HA1-ELP. Plates coated with 1 µg either ^{Nt}H5-ELP or ^{Nt}HA1-ELP per well were incubated with $2x10^{-4}$ dilution of chicken sera. Then the plates were incubated with a rabbit anti-chicken IgY antibody-conjugated alkaline phosphatase. Enzyme activity was visualized using pNPP. The absorbance signal was mearsured at 405 nm. A single dot indicates an ELISA value of a single chicken. Bars present the average value of the test groups.

III.8. Immunogenicity in mice

III.8.1. High immunogenicity of trimeric hemagglutinins

Since neutralizing antibodies were not induced after chickens were vaccinated with the single dose of the purified monomeric hemagglutinins (^{Nt}H5-ELP and ^{Nt}HA1-ELP). Therefore, in the second animal experiment, mice were vaccinated four times with 10 and 50 µg of Freund adjuvanted monomeric hemagglutinins (^{Nt}H5 and ^{Nt}H5-ELP). Mice were also vaccinated four times with 10 and 50 µg of Freund adjuvanted trimeric hemagglutinins (^{Nb}(H5pII)3 and ^{Nb}(H5pII-ELP)3) (Fig. III-30B). The purpose of this experiment is to determine whether monomeric hemagglutinins or trimeric hemagglutinins could induce neutralizing antibodies.

Mouse sera were only collected for detection of target-specific antibody responses by ELISA and neutralizing antibodies by HI tests after the third and fourth immunization (Fig. III-30B). To measure the immune responses induced by the ^{Nb}(H5pII-ELP)3 trimer and ^{Nt}H5-ELP monomer by ELISA, 300 ng/well of ^{Nt}H5-ELP were absorbed on plates. Similarly, 300 ng/well of ^{Nt}H5 were absorbed on plates to compare immune responses induced by ^{Nb}(H5pII)3 and ^{Nt}H5. Then the same dilution ($2x10^{-4}$ fold) of individual mouse sera from mice vaccinated with 10 and 50 µg of ^{Nb}(H5pII-ELP)3, ^{Nt}H5-ELP, ^{Nb}(H5pII)3 and ^{Nt}H5 were applied. The addition of the secondary anti-mouse IgG antibody alkaline phosphatase conjugate allowed to quantify target specific mouse antibodies.

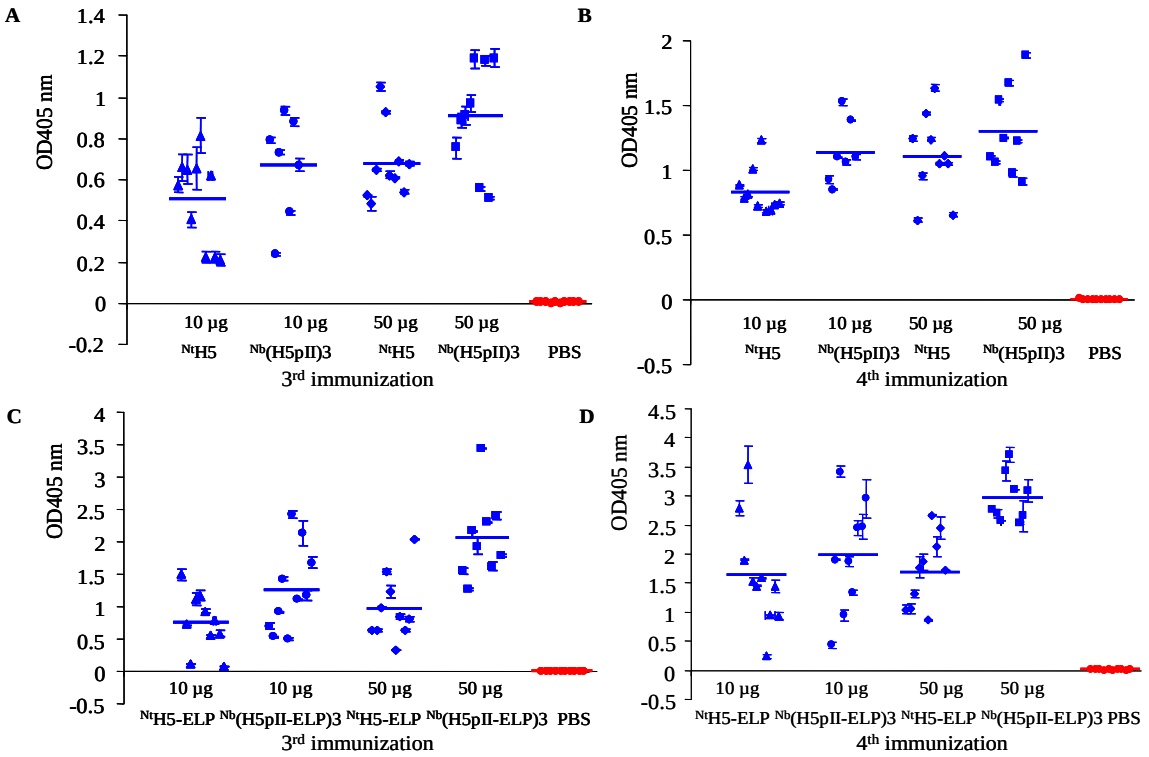

Figure III-32. Mouse antibody responses, as determined by ELISA. ^{Nt}H5 and ^{Nb}(H5pII)3 antibody responses after the 3rd (A) and 4th immunization (B). ^{Nt}H5-ELP and ^{Nb}(H5pII-ELP)3 antibody responses after the 3rd (C) and 4th immunization (D). Plates coated with 300 ng of ^{Nt}H5 (A and B) or ^{Nt}H5-ELP (C

and D) per well were incubated with $2x10^{-4}$ dilution of mouse sera. The plates were then incubated with a rabbit anti-mouse IgG alkaline phosphatase conjugate. Enzyme activity was determined using pNPP. The absorbance signal was mearsured at 405 nm. A single dot indicates an ELISA value of a single mouse. Bars present the average value of the test groups.

As shown in Fig. III-32, all mice immunized with 10 and 50 µg of ^{Nb}(H5pII-ELP)3, ^{Nt}H5-ELP, ^{Nb}(H5pII)3 and ^{Nt}H5 mounted target specific immune responses, while the control mouse sera did not show any immune responses. The immune responses were dose-dependent and augmented by the fourth immunization (Fig. III-32). Moreover, mean absorbance values of mouse sera from mice vaccinated with the ^{Nb}(H5pII)3 and ^{Nb}(H5pII-ELP)3 trimeric hemagglutinins were always higher than those of mouse sera from mice that vaccinated with the ^{Nt}H5 and ^{Nt}H5-ELP monomeric hemgglutinins, respectively (Fig. III-32).

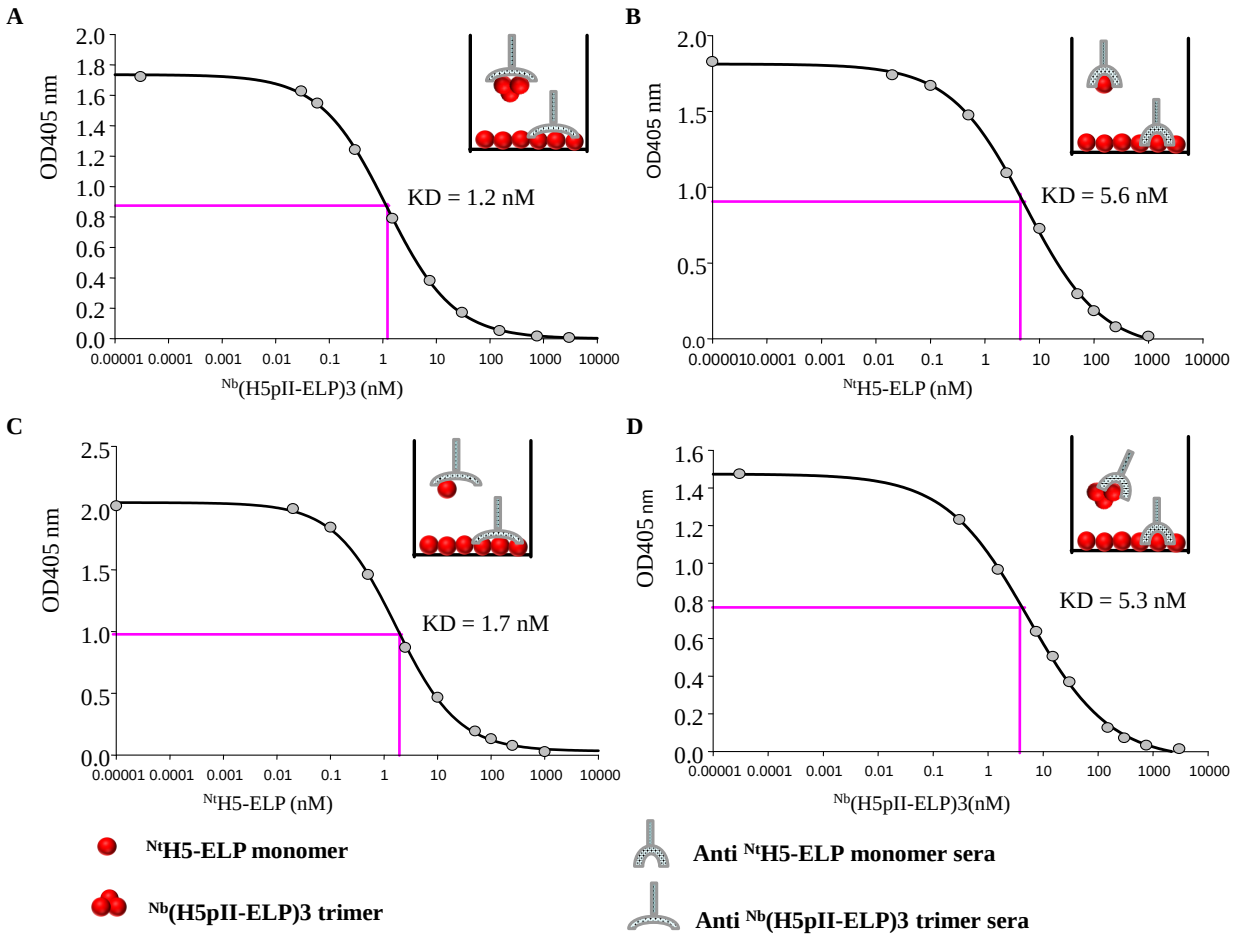

Figure III-33. Robustness of the trimeric hemagglutinins in immune responses, as determined by competitive ELISAs. Binding activity of the anti ^{Nb}(H5pII-ELP)3 trimer serum and ^{Nb}(H5pII-ELP)3 (A), the anti ^{Nt}H5-ELP monomer serum and ^{Nt}H5-ELP (B), the anti ^{Nb}(H5pII-ELP)3 trimer serum and ^{Nt}H5-ELP (C) and the anti ^{Nt}H5-ELP monomer serum and ^{Nb}(H5pII-ELP)3 (D). Plates were coated with the purified ^{Nt}H5-ELP (300 ng per well). Various concentrations of pre-determined quantities of recombinant antigens mixed with specific dilution of mouse sera ($2x10^{-4}$) in 1% w/v BSA in PBST were added to each well. Antigen-antibody complexes were visualized by treatment with rabbit anti-

mouse IgG alkaline phosphatase conjugate. Enzyme activity was determined using pNPP. The absorbance signal was mearsured at 405 nm. The KD values were calculated from the competition curves at 50% inhibition from the molarities of free soluble monomeric antigens.

To further compare the immunogenicity of the trimeric and monomeric hemagglutinins, a competitive ELISA approach was used. Mouse sera induced by trimeric ^{Nb}(H5pII-ELP)3 and monomeric ^{Nt}H5-ELP were selected and the trimeric ^{Nb}(H5pII-ELP)3 and monomeric ^{Nt}H5-ELP antigens were used as competitors. The binding affinities of the combinations between the anti ^{Nb}(H5pII-ELP)3 serum and ^{Nb}(H5pII-ELP)3, the anti ^{Nt}H5-ELP serum and ^{Nt}H5-ELP, the anti ^{Nb}(H5pII-ELP)3 serum and ^{Nt}H5-ELP, the anti ^{Nt}H5-ELP serum and ^{Nb}(H5pII-ELP)3 were calculated and shown in Fig. III-33, respectively. Dissociation constants (KD) of the anti trimer serum and trimeric antigen complex, the anti trimer serum-monomer antigen complex were 1.2 and 1.7 nM, respectively (Fig. III-33A and C). KDs of the anti monomeric serum and monomer antigen complex, the anti monomeric serum-trimer antigen complex were 5.6 and 5.3 nM, respectively (Fig. III-32B and D). These competitive ELISA analyses indicated that the binding of antibodies induced by trimeric hemagglutinins was generally stronger than that of antibodies induced hemagglutinin monomers. Taking the ELISA and competitive ELISA analyses into consideration, the trimeric hemagglutinins were more immunogenic than the monomeric hemagglutinins.

The neutralizing antibodies induced by plant-produced hemagglutinins were measured by the HI assay. In the mouse groups which were vaccinated with 10 µg of the ^{Nb}(H5pII)3 and ^{Nb}(H5pII-ELP)3 trimeric hemagglutinins, 100% of them had serum HI titers ≥ 64 against homologous VLPs A/Hatay/2004/(H5N1), while 89% of mice that received 50 µg of the ^{Nb}(H5pII)3 and ^{Nb}(H5pII-ELP)3 trimeric hemagglutinins had HI titers ≥ 64 against homologous VLPs after the 3rd immunization (Fig. III-34A and B, Table III-12). Following the 4th immunization, all sera from mice vaccinated with 10 and 50 µg of the ^{Nb}(H5pII)3 and ^{Nb}(H5pII-ELP)3 trimeric hemagglutinins had HI titers ≥ 64 against homologous VLPs (Fig. III-34C and D, Table III-12), while almost sera from mice vaccinated with 10 and 50 µg of ^{Nt}H5 and ^{Nt}H5-ELP had HI titer ≤ 32. One animal (10%) that received 10 µg of ^{Nt}H5 had a HI titer of 64 after the 3rd immunization (red curve in Fig. III-33B, Table III-12). The 4th immunization of ^{Nt}H5 and ^{Nt}H5-ELP monomeric hemagglutinins augmented the HI titers, whereas the majority of sera (40-90%) in all four groups had HI titers of 32. 10-20% of sera had HI antibody of 64 (Fig. III-34C and D, Table III-12). A serum HI titer ≥ 40 is correlated with vaccine-induced protection in humans (Hobson *et al.*, 1972). These HI titer results indicate that the ^{Nb}(H5pII)3 and ^{Nb}(H5pII-ELP)3 trimeric hemagglutinins were highly able to

induce neutralizing antibodies against homologous VLPs, whereas the ^{Nt}H5 and ^{Nt}H5-ELP monomeric hemagglutinins induce these antibodies poorly.

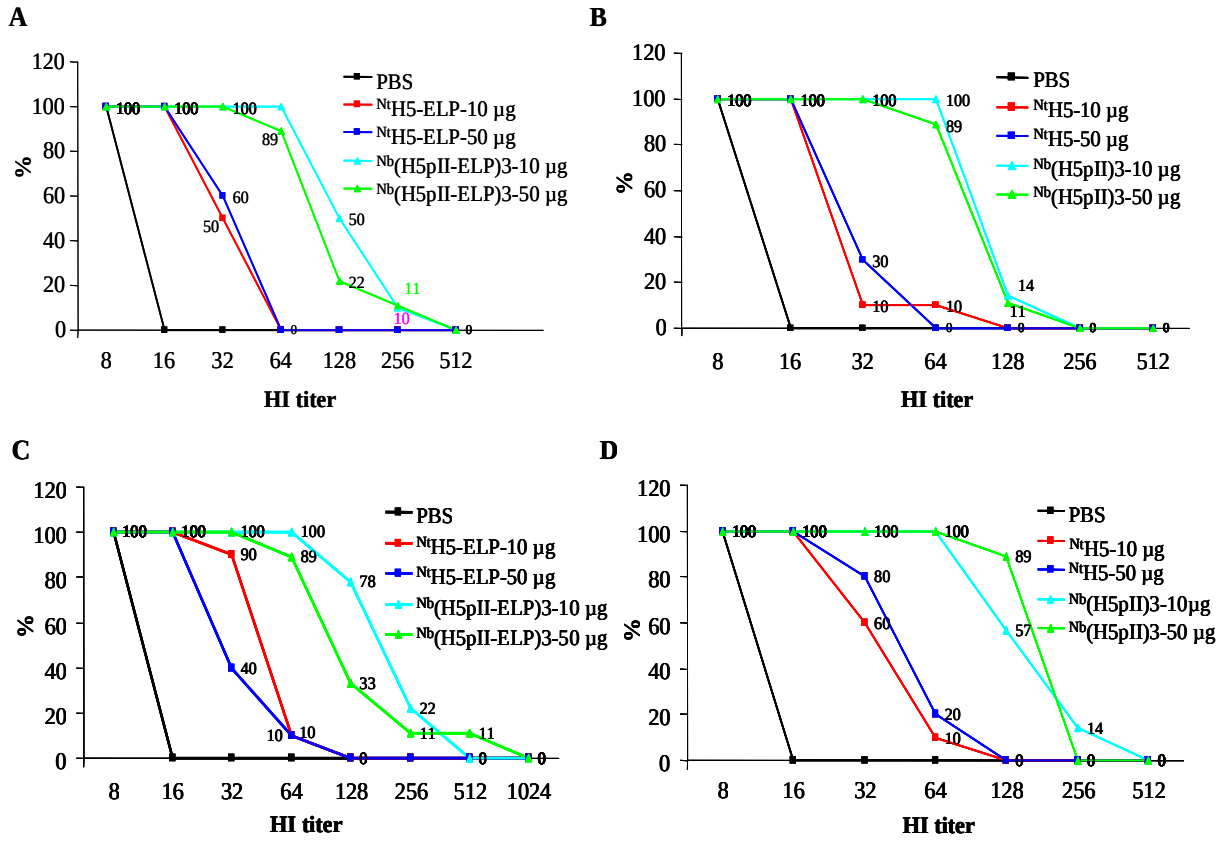

Figure III-34. Serum HI titers against homologous VLPs (clade 1). Hemagglutination inhibition titers of sera from mice immunized three times with 10 and 50 µg/dose of ^{Nt}H5-ELP and ^{Nb}(H5pII-ELP)3 (A), three times with 10 and 50 µg/dose of ^{Nt}H5 and ^{Nb}(H5pII)3 (B), four times with 10 and 50 µg/dose of ^{Nt}H5-ELP and ^{Nb}(H5pII-ELP)3 (C) and four times with 10 and 50 µg/dose of ^{Nt}H5 and ^{Nb}(H5pII)3 (D). HI titers were expressed as the reciprocal of the highest dilution of serum which inhibited the hemagglutination of four hemagglutination units of homologous VLPs.

Table III-12. Serum HI titers against homologous VLPs and heterologous strain. 1st, 2nd, 3rd, 4th indicate vaccination times of mice. Homologous VLPs: hemagglutinin-based virus-like particles were produced in plant cells using hemagglutinin from A/Hatay/2004(H5N1) strain. Heterologous strain: inactivated virus rg A/swan/Germany/R65/2006 (H5N1). AbISCO adjuvant is the 3rd generation of immune stimulating complex Matrix M™, produced by the ISCONOVA company. A serum HI titer ≥ 40 is correlated with vaccine-induced protection in humans. All positive serum HI titers shown in this table have HI titers ≥ 64.

Antigen	Dose	Homologous VLPs		Heterologous strain	
		3rd	4th	3rd	4th
Freund's adjuvant					
NtH5-ELP	50 µg	0/10	1/10 (10%)	0/10	0/10
	10 µg	0/10	1/10 (10%)	0/10	0/10
NtH5	50 µg	0/10	2/10 (20%)	0/10	0/10
	10 µg	0/10	1/10 (10%)	0/10	1/10
Nb(H5pII-ELP)3	50 µg	8/9 (89%)	8/9 (89%)	1/9 (11%)	2/9 (22%)
	10 µg	9/9 (100%)	9/9	3/9 (33%)	4/9 (44%)
Nb(H5pII)3	50 µg	8/9 (89%)	9/9	1/9 (11%)	1/9 (11%)
	10 µg	7/7	7/7	1/7 (14%)	1/7 (14%)
PBS		0/10	0/10	0/10	0/10

AbISCO adjuvant

Antigen	Dose	Homologous VLPs			Heterologous strain		
		1st	2nd	3rd	1st	2nd	3rd
Nb(H5pII-ELP)3	10 µg	0/10	9/10	10/10	0/10	2/10	4/10
Nb(H5pII)3	10 µg	0/10	9/10	10/10	0/10	1/10	2/10

III.8.2. ISCOM-based Matrix M™ adjuvant: A new potential adjuvant

The HI results in section III.8.1 clearly show that trimeric hemagglutinins could induce functional antibodies against homologous VLPs after the third immunization. These results prompted us to determine how many immunization times are required to elicit neutralizing antibodies. Furthermore, particulate antigens could induce higher immune responses than soluble antigens due to antigen improvement uptake by APCs. In order to assess the immunogenicity of the trimeric hemagglutinins, an abISCO adjuvant, one of the ISCOM Matrix M™ adjuvant with particle of approximately 40 nm in diameter (Lövgren Bengtsson *et al*., 2011) was used. To this end, three mouse groups (ten mice/group) were vaccinated three times with 10 µg of abISCO adjuvanted Nb(H5pII)3, Nb(H5pII-ELP)3 and PBS. One week after each immunization, sera from three groups were collected for ELISA and HI titer analyses (Fig. III-30C). As shown in Fig. III-35, mice primed with Nb(H5pII)3 and Nb(H5pII-ELP)3 had poor specific serum IgG responses to NtH5 and NtH5-ELP, respectively. Following the first

antigen boost, higher serum IgG titers were detected from both groups vaccinated with ^{Nb}(H5pII)3 and ^{Nb}(H5pII-ELP)3. After the second boost, ^{Nt}H5-ELP and ^{Nt}H5 specific IgG immune responses dramatically increased, respectively (Fig. III-35A and B).

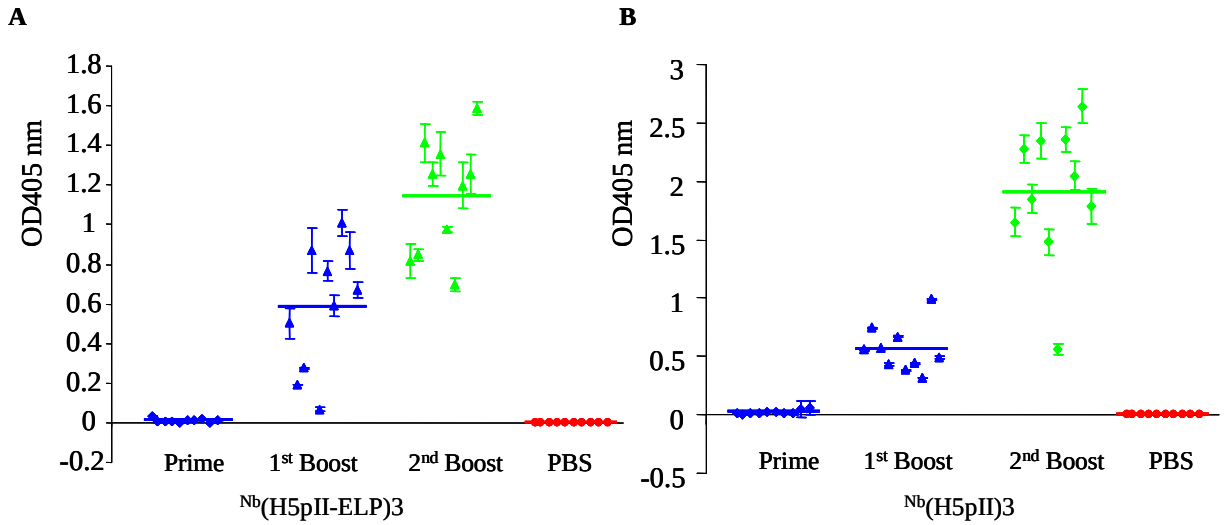

Figure III-35. Mouse immune responses after priming, 1st boost and 2nd boost with abISCO adjuvanted ^{Nb}(H5pII-ELP)3 and ^{Nb}(H5pII)3. (A) Mouse sera from mice vaccinated with abISCO adjuvanted ^{Nb}(H5pII-ELP)3. (B) Mouse sera from mice vaccinated with abISCO adjuvanted ^{Nb}(H5pII)3. Plates were coated with 300 ng of purified ^{Nt}H5-ELP or ^{Nt}H5 per well. Specific dilution of mouse sera ($2x10^{-4}$) in 1% w/v BSA in PBST was added to each well. Then the plates were incubated with a rabbit anti-mouse IgG alkaline phosphatase conjugate. Enzyme activity was visualized using pNPP. The absorbance signal was mearsured at 405 nm. A single dot indicates an ELISA value of a single mouse. Bars present the average value of the test groups.

These total immune responses correlated well with the HI titers against homologous VLPs (Fig. III-37). After priming, all sera from a control group had HI titers as low as 8, while 100% and 50% of sera from mice vaccinated with ^{Nb}(H5pII-ELP) and ^{Nb}(H5pII)3 had HI titer of 16, respectively. After the first antigen boost, 90% of mice immunized with abISCO adjuvanted ^{Nb}(H5pII)3, ^{Nb}(H5pII-ELP)3 had HI titers ≥ 64 against homologous VLPs with a maximum HI titer of 256 and a minimum HI titer of 32. Following the second antigen boost, all animals vaccinated with abISCO adjuvanted ^{Nb}(H5pII)3 and ^{Nb}(H5pII-ELP)3 had HI serum antibody titers ≥ 64. Maximum HI titer was 512 (10%) in the both vaccinated groups (Fig. III-37 and Table III-12). In general, HI titers were augmented after each the antigen boost.

To compare effects of two adjuvants (AbISCO and Freund's adjuvants) on the immune responses, sera from mice vaccinated three times with 10 µg of abISCO and Freund adjuvanted ^{Nb}(H5pII)3 and ^{Nb}(H5pII-ELP)3 were used to determine the ^{Nt}H5 specific IgG by ELISA analyses. The ELISA results show that mean absorbance values of sera from mice vaccinated with the abISCO adjuvanted ^{Nb}(H5pII-ELP)3 and ^{Nb}(H5pII)3 were always significantly higher

than those of sera from mice vaccinated with the Freund adjuvanted ^{Nb}(H5pII-ELP)3 and ^{Nb}(H5pII)3, respectively (Fig. III-36A and B).

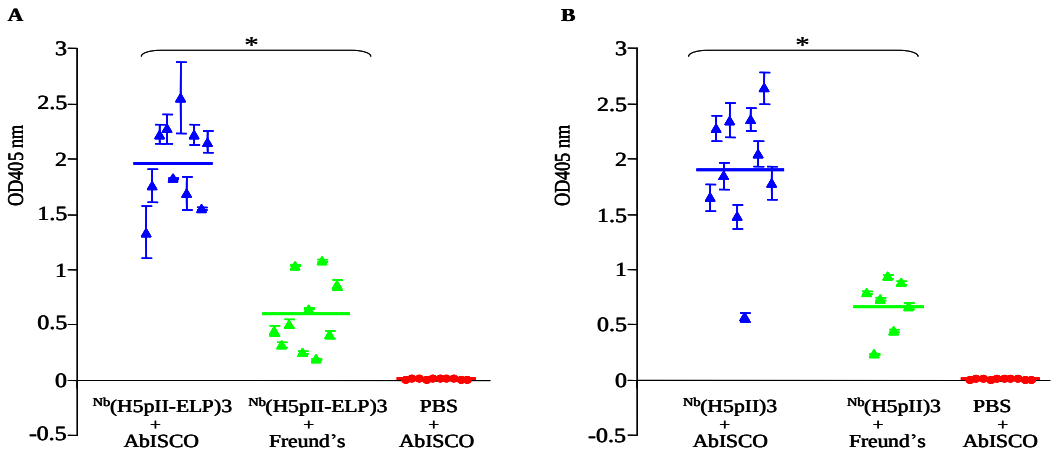

Figure III-36. Robustness of an abISCO adjuvant, as determined by ELISAs. Sera from mice vaccinated with 10 µg of abISCO, Freund adjuvanted ^{Nb}(H5pII-ELP)3 (A) and ^{Nb}(H5pII)3 (B). Plates were coated with 300 ng of purified ^{Nt}H5 per well. Specific dilution of mouse sera ($2x10^{-4}$) in 1% w/v BSA in PBST was added to each well. Then the plates were incubated with a rabbit anti-mouse IgG alkaline phosphatase conjugate. Enzyme activity was determined using pNPP. The absorbance signal was mearsured at 405 nm. A single dot indicates an ELISA value of a single mouse. Bars present the average value of the test groups. * indicates $p < 0.05$ determined using Student's t-test.

Consequently, HI serum antibody titers of mice vaccinated with abISCO adjuvanted ^{Nb}(H5pII)3 and ^{Nb}(H5pII-ELP)3 were higher than those of mice vaccinated with Freund adjuvanted ^{Nb}(H5pII)3 and ^{Nb}(H5pII-ELP)3, respectively (Fig. III-38 and Fig. III-41). Sera from mice vaccinated with abISCO adjuvanted ^{Nb}(H5pII)3 and ^{Nb}(H5pII-ELP)3 had geometric mean HI titers of approximately 207 and 147, repectively, whereas mice vaccinated with Freund adjuvanted ^{Nb}(H5pII)3 and ^{Nb}(H5pII-ELP)3 had geometric mean HI titers of approximately 71 and 97, repectively.

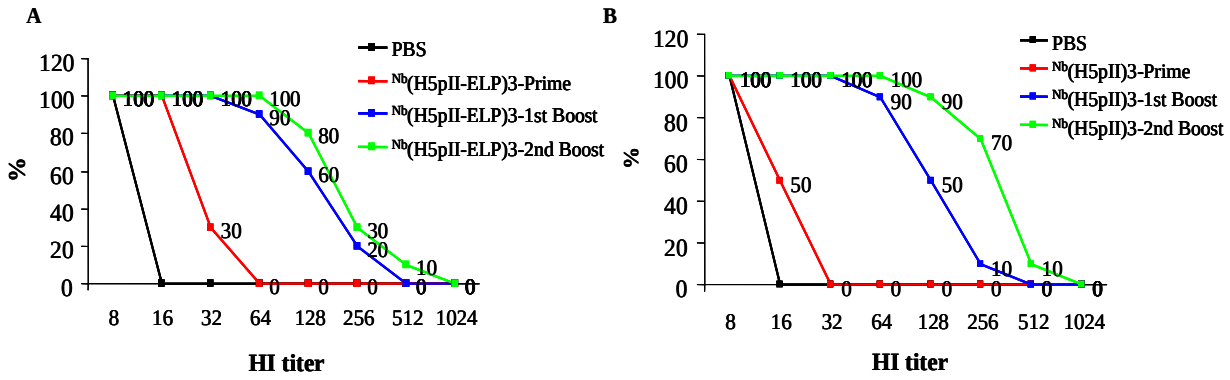

Figure III-37. HI titers of mouse sera after priming, 1st boost and 2nd boost with abISCO adjuvanted ^{Nb}(H5pII-ELP)3 and ^{Nb}(H5pII)3. Sera from mice vaccinated with 10 µg of abISCO adjuvanted ^{Nb}(H5pII-ELP)3 (A) and ^{Nb}(H5pII)3 (B). HI titers were expressed as the reciprocal of the

highest dilution of serum which inhibited the hemagglutination of four hemagglutination units of homologous VLPs.

In brief, these results indicate that (1) neutralizing antibodies against homologous VLPs were induced after two immunizations of trimeric hemagglutinins and (2) the third generation immune stimulating complex Matrix M^{TM}, abISCO adjuvant, not only enhanced significantly immune responses but also elicited stronger neutralizing antibodies against homologous VLPs.

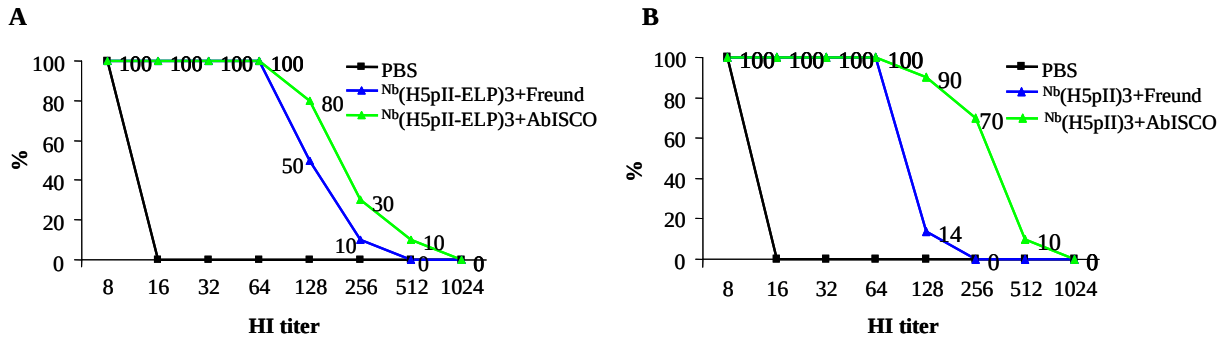

Figure III-38. HI titers against homologous VLPs of sera from mice vaccinated three times with abISCO and Freund adjuvanted trimeric hemagglutinins. Sera from mice vaccinated three times with 10 µg of abISCO, Freund adjuvanted ^{Nb}(H5pII-ELP)3 (A) and ^{Nb}(H5pII)3 (B). HI titers were expressed as the reciprocal of the highest dilution of serum which inhibited the hemagglutination of four hemagglutination units of homologous VLPs.

III.8.3. Cross-reactive HI antibody responses

Since HP H5N1 avian influenza viruses continue to evolve, different variants now exist. The different H5N1 viruses can be clustered genetically into 10 distinct clades (and subclades). It is ideal that candidate pandemic vaccines can induce broadly cross-reactive antibody responses. In order to determine cross-reactive antibody responses, neutralizing antibodies against the heterologous strain rg A/swan/Germany/R65/2006(H5N1) (clade 2.2.2) were measured by HI assays.

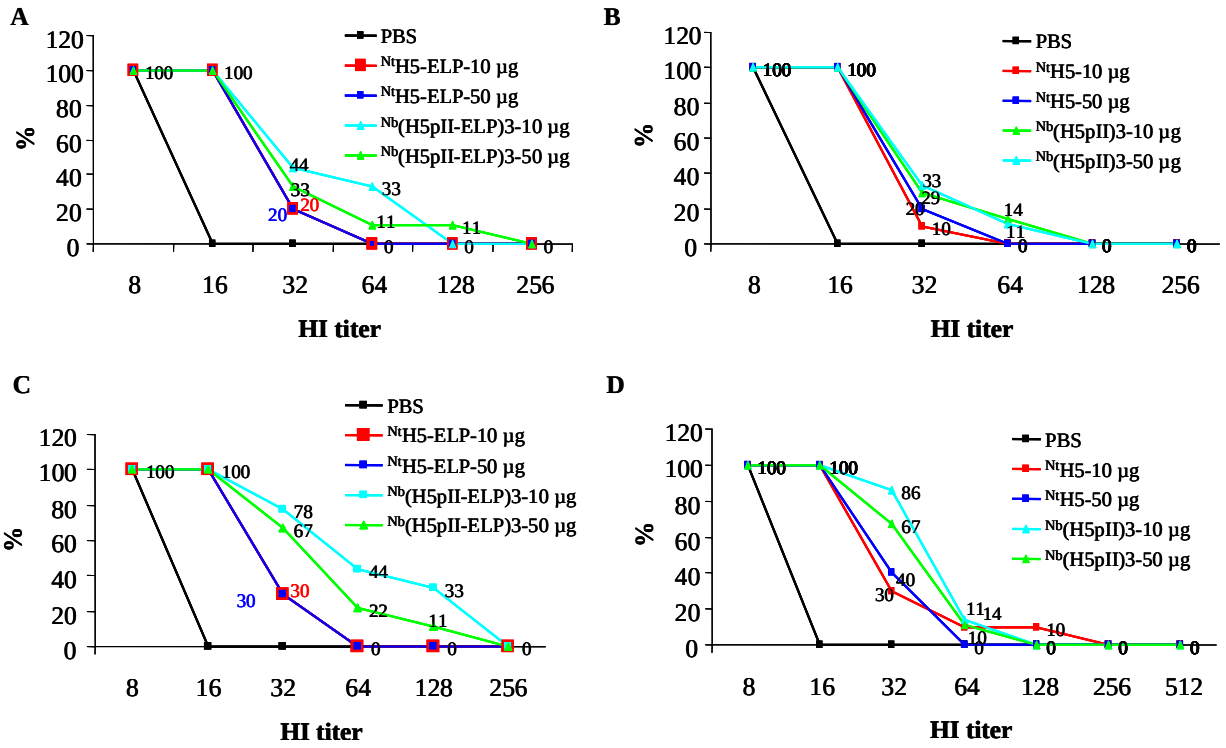

Figure III-39. Serum HI titers against heterologous strain (clade 2.2.2). Hemagglutination inhibition titers of sera from mice immunized three times with 10 and 50 µg/dose of the Freund adjuvanted ^{Nt}H5-ELP and ^{Nb}(H5pII-ELP)3 (A), three times with 10 and 50 µg/dose of the Freund adjuvanted ^{Nt}H5 and ^{Nb}(H5pII)3 (B), four times with 10 and 50 µg/dose of the Freund adjuvanted ^{Nt}H5-ELP and ^{Nb}(H5pII-ELP)3 (C) and four times with 10 and 50 µg/dose of the Freund adjuvanted ^{Nt}H5 and ^{Nb}(H5pII)3 (D). HI titers were expressed as the reciprocal of the highest dilution of serum which inhibited the hemagglutination of four hemagglutination units of heterologous strain rg A/swan/Germany/R65/2006(H5N1).

After the third immunization, no mice that received the Freund adjuvanted ^{Nt}H5 and ^{Nt}H5-ELP monomeric hemagglutinins had HI titers against heterologous strain higher than 32 (Fig. III-39, Table III-12), while the seroprotection with HI titers ≥ 64 were detected in 30% and 11% of mice vaccinated with 10 and 50 µg of Freund adjuvanted ^{Nb}(H5pII-ELP)3, respectively (Fig. III-39A, Table III-12) and 14% and 11% of mice vaccinated with 10 and 50 µg of Freund-adjuvanted ^{Nb}(H5pII)3 trimeric hemagglutinin, respectively (Fig. III-39B, Table III-12). Following the fourth antigen immunization, mice vaccinated with the ^{Nb}(H5pII-ELP)3 and ^{Nb}(H5pII)3 trimeric hemagglutinins with HI titer of 32 significantly increased in comparison to the third immunization, while mouse groups vaccinated with the monomeric hemagglutinins slightly increased (Fig. III-39C-D, Table III-12). Seroprotection (indicates HI titer ≥ 64) from mice vaccinated with ^{Nb}(H5pII-ELP)3 also increased (Fig. III-39C), but seroprotection did not change in groups vaccinated with ^{Nb}(H5pII)3 after the fourth immunization (Fig. III-39D, Table III-12). 44% and 22% of animals that vaccinated with 10

and 50 µg of ^{Nb}(H5pII-ELP)3 showed seroprotection against the heterologous strain after the fourth immunization, respectively (Fig. III-39C, Table III-12). These values were higher than that in groups vaccinated with 10 and 50 µg of ^{Nb}(H5pII)3 (14% and 11% in Fig. III-39D, Table III-12).

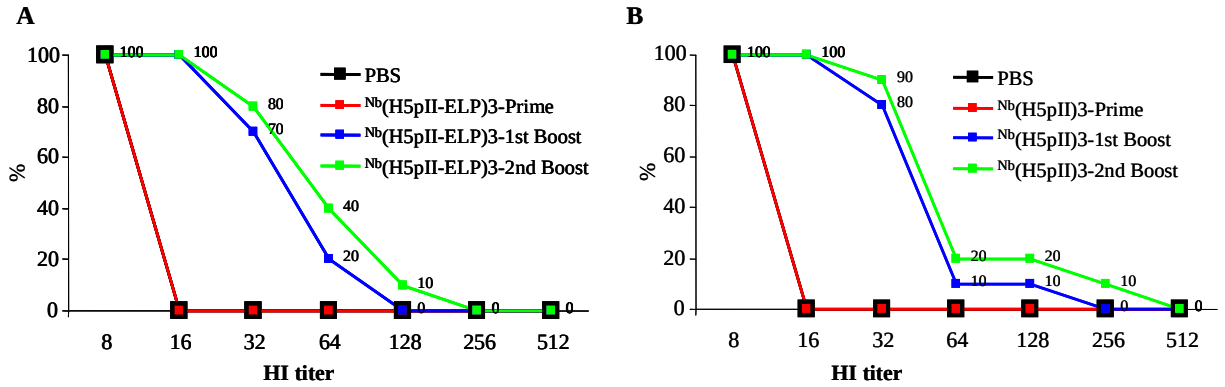

Figure III-40. HI titers against heterologous strain of mouse sera after priming, 1st boost and 2nd boost with abISCO adjuvanted ^{Nb}(H5pII-ELP)3 and ^{Nb}(H5pII)3. Sera from mice vaccinated with 10 µg of abISCO adjuvanted ^{Nb}(H5pII-ELP)3 and ^{Nb}(H5pII)3 (B). HI titers were expressed as the reciprocal of the highest dilution of serum which inhibited the hemagglutination of four hemagglutination units of the heterologous strain rg A/swan/Germany/ R65/2006(H5N1).

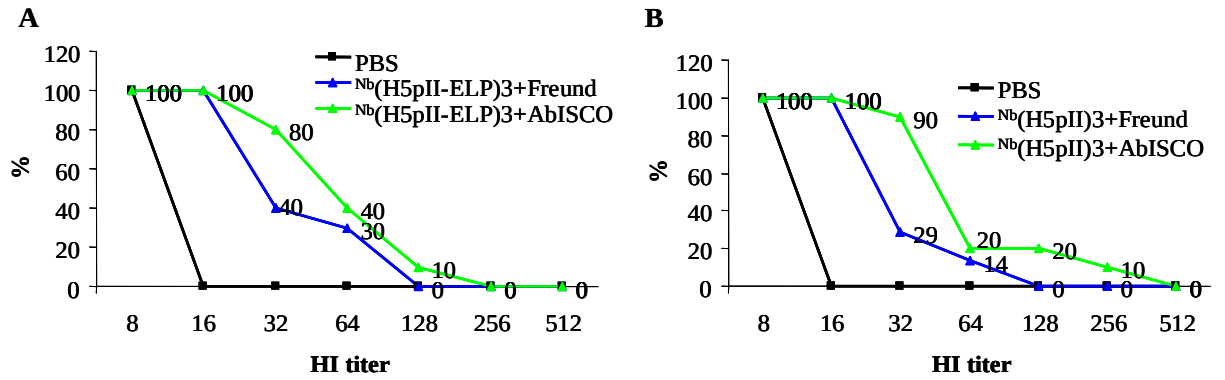

Figure III-41. HI titers against heterologous strain of sera from mice vaccinated three times with abISCO and Freund adjuvanted trimeric hemagglutinins. Sera from mice vaccinated with 10 µg of abISCO, Freund adjuvanted ^{Nb}(H5pII-ELP)3 (A) and ^{Nb}(H5pII)3 (B). HI titers were expressed as the reciprocal of the highest dilution of serum which inhibited the hemagglutination of four hemagglutination units of heterologous strain rg A/swan/Germany/R65/2006(H5N1).

Seroprotection against the heterologous strain of sera from mice vaccinated with abISCO adjuvanted ^{Nb}(H5pII-ELP)3 and ^{Nb}(H5pII)3 trimeric hemagglutinins were detected after the first antigen boost forwards (Fig. III-40, Table III-12). After the second antigen boost, seroprotection was augmented from 20% (after the first boost) to 40% in group vaccinated with 10 µg of abISCO adjuvanted ^{Nb}(H5pII-ELP)3 (Fig. III-40A, Table III-12). Similarly, increasing seroprotection was also observed in the group vaccinated with 10 µg of abISCO adjuvanted ^{Nb}(H5pII)3 (after the second boost from 10% to 20%) (Fig. III-40B, Table III-12).

HI titer analyses showed that seroprotection HI titers from animals vaccinated with abISCO adjuvanted trimeric hemagglutinins were always higher than those from animals vaccinated with Freund adjuvanted trimeric hemagglutinins (Fig. III-41). 40% of animals with seroprotection against the heterologous strain were observed in group vaccinated with 10 µg of abISCO adjuvanted ^{Nb}(H5pII-ELP)3, while 30% of animals were detected in group vaccinated with 10 µg of Freund adjuvanted ^{Nb}(H5pII-ELP)3 (Fig. III-41A). Similarly, 20% and 14% seroprotections against the heterologoust strain were detected in groups vaccinated with 10 µg of abISCO and Freund adjuvanted ^{Nb}(H5pII)3, respectively (Fig. III-41B, Table III-12).

In summary, ^{Nb}(H5pII)3 and ^{Nb}(H5pII-ELP)3 trimeric hemagglutinins were able to induce neutralizing antibodies against heterologous strain, whereas ^{Nt}H5 and ^{Nt}H5-ELP monomeric hemagglutinins induce these antibodies poorly. Neutralizing antibodies against heterologous strain were detected after two immunizations. The third generation immune stimulating complex Matrix M^{TM}, abISCO adjuvant, could elicit more neutralizing antibodies against the heterologous strain in comparison to Freund's adjuvant.

III.8.4. Immunogenicity of the 100xELP tag

Since hemagglutinin was expressed in plant cells as an ELP fusion protein, the immunogenicity of ELP tag and its hemagglutinin fusion partner was assessed. Hemagglutinin and ELP tag specific antibodies in mouse sera after the third immunization from mice vaccinated with 10 µg of abISCO adjuvanted ^{Nb}(H5pII-ELP)3 were detected by direct ELISA.

For this purpose, 100xELP tag containing his and c-myc tags was purified by affinity chromatography (Fig. VII-1). Because recombinant ^{Nb}(H5pII-ELP)3 contains both his and c-myc tags, mouse antibodies induced by abISCO adjuvanted ^{Nb}(H5pII-ELP)3 were tested for cross-reaction with his and c-myc tags. A single chain fragment variable antibody against regulatory component of ABA receptor (aRCAR) which contains his and c-myc tags was used as a control. Hemagglutinin without ELP, aRCAR and ELP tag were coated separately on plates. The plates were then incubated with same serum dilution ($2x10^{-4}$). The plates were subsequently incubated with rabbit anti-mouse IgG alkaline phosphatase conjugate and enzymatic activity was measured at 405 nm.

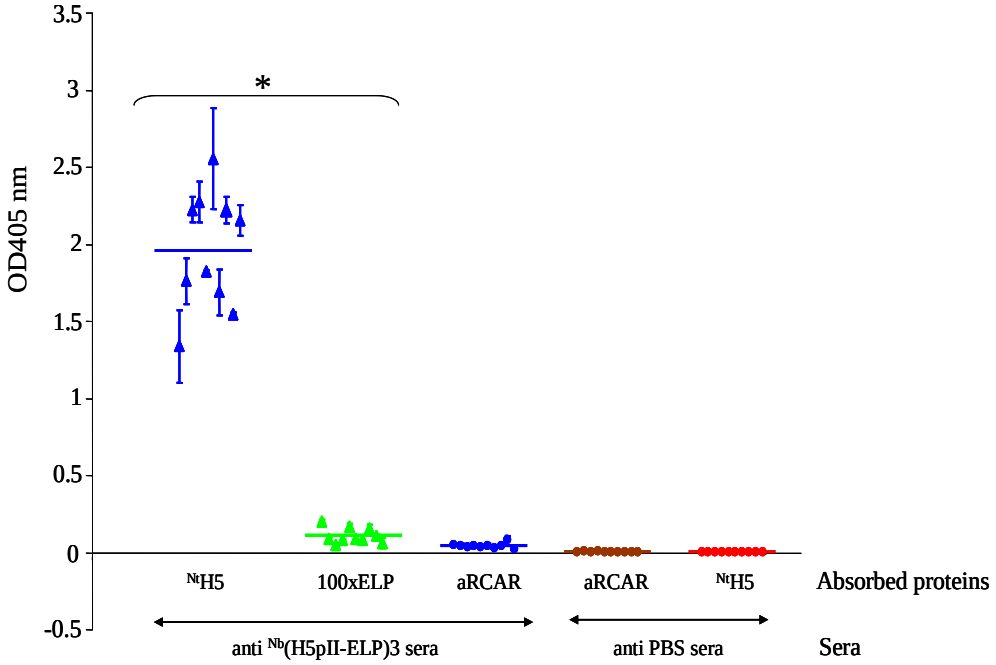

Figure III-42. Immune responses against the 100xELP tag and ^{Nt}H5. The same amount (300 ng/well) of ^{Nt}H5, 100xELP tag and aRCAR proteins were coated on plates. The anti abISCO adjuvanted ^{Nb}(H5pII-ELP)3 sera were applied on the plates. The plates were then incubated with a rabbit anti-mouse IgG alkaline phosphatase conjugate. Enzyme activity was determined using pNPP. The absorbance signal was mearsured at 405 nm. A single dot indicates an ELISA value of a single mouse. Bars present the average value of the test groups. * indicates $p < 0.05$ determined using Student's t-test.

Fig. III-42 shows that antibodies induced by abISCO adjuvanted ^{Nb}(H5pII-ELP)3 were strongly specific for ^{Nt}H5 hemagglutinin. These antibodies also reacted to a very low extend with aRCAR protein, while control sera did not. These results indicate that the ^{Nb}(H5pII-ELP)3 induced antibodies could recognize the c-myc and 6x his tags present within aRCAR protein. No significant immune responses against 100xELP tag were observed in comparison to control. Taken together, these results imply that 100xELP tag is low immunogenic and immune responses mainly target on hemagglutinin.

IV. Discussion

Influenza is an infectious disease of the respiratory tract. This is caused by virus that was responsible for one of the most destructive outbreaks of any contagious disease in history. In the last century, there were three influenza pandemics caused by the influenza H1N1 (1918), H2N2 (1957) and H3N2 (1968) viruses (Reid *et al.*, 1999; Reid *et al.*, 2001; Stevens *et al.*, 2004). Currently, the worldwide emergence of the novel influenza A H5N1 with high mortality rates 59% (332 deaths/566 human cases) has raised concerns that this virus subtype could adapt to transmit effectively among humans and cause a next pandemic (section I-2).

Influenza vaccines have been produced traditionally in ECEs over 60 years. The egg-based technology for producing influenza vaccine now reaches its limits in terms of vaccine production time and capacity. This technology will not allow manufacturing to have a rapid response to the increasing vaccine demands for a pandemic situation (Gregersen *et al.*, 2011). Many attemps are under way to develop rapid, efficient and reliable vaccine production technologies (Lambert and Fauci, 2010). Among these, influenza production vaccines using alternative substrates is a promising approach followed by influenza vaccine manufactures. Alternative substrates could be Vero cells to produce the inactivated whole virus vaccines (Ehrlich *et al.*, 2008), or mammalian cell culture (Cornelissen *et al.*, 2010; Du *et al.*, 2011), insect cells (Treanor *et al.*, 2001; Safdar and Cox, 2007) and *E. coli* (Khurana *et al.*, 2010; Khurana *et al.*, 2011; Biesova *et al*., 2009) to produce influenza subunit vaccines.

More recently, expressing recombinant proteins in plants has been proposed an attractive way since plant expression systems offer a number of potential advantages over the classical expression systems based on bacterial, microbial and animal cells (section I.2; for review see Fischer and Emans, 2000; Ma *et al.*, 2005; Twyman, 2003). Since 1990, when the first antigen was expressed in tobacco plant, numerous recombinant proteins were expressed in plant expression systems (for review see Floss *et al*., 2007; Twyman *et al*., 2005). Here we ask the question whether plants could be a suitable tool for production of influenza vaccines. The question includes not only the expression level of influenza antigens but also their recovery and purification. To answer these questions, the influenza antigens eliciting protective antibodies, HA and NA, were expressed in tobacco leaves and seeds. The central topic of this study is to optimize expression of the functional hemaglutinin and neuraminidase in tobacco plants, to enrich these proteins from plant materials by ITC and to immunogically evaluate them in animals.

IV.1. Plant-produced influenza antigens

The transient expression system has emerged as a rapid and useful method to analyse genes and gene products in plants. It presents a feasible tool for testing accumulation of the foreign proteins in plant cells before processing to develop stably transformed plants. In this study, *N. benthaminiana* was used to check the feasible expression of hemagglutinin and neuraminidase and to produce these proteins as influenza candidate vaccines. In order to accumulate efficiently hemagglutinin in the ER, the TMD and CT domain should be removed. The ectodomain of hemagglutinin (^{Nb}H5) and its subunits (^{Nb}HA1 and ^{Nb}HA2) were clearly detectable (Fig. III-5A). These results were similar to the findings observed by Shoji and co-workers and Spitsin and co-workers who reported that extracellular hemagglutinins and HA1 domains of the H5N1 hemagglutinin were produced efficiently in *N. benthamiana* (Shoji *et al*., 2009a and 2009b; Spitsin *et al*., 2009). Full length hemagglutinin (HA0ER) including the TMD and CT was not expressed (Fig. III-5A). This protein was directed into the ER and stored in this compartment by the KDEL signal at the C-terminus (Fig. III-3B). These results indicated that HA0ER was not efficiently accumulated in the ER because it contained two components with opposite functions. Influenza hemagglutinin TMD acts as a membrane-anchored domain directing the hemagglutinin to the apical plasma membrane (Nayak *et al.*, 2004), while the KDEL motif at the C-terminus of recombinant protein has been shown to be necessary to stabilize and accumulate recombinant proteins in the ER lumen (Wandelt *et al.*, 1992). In contrast with HA0ER, the full hemagglutinin (^{Nb}H5-VLP) protein containing only the TMD and CT was successfully expressed in plant cells (Fig. III-5B). This protein was proved that it could form virus-like particles (Fig. III-28). This result was similar with the findings observed by D'Aoust and co-workers who reported that the full hemagglutinin of the A/Indonesia/5/05(H5N1) strain with its native signal peptide, TMD and CT was expressed in *N. benthamiana* under control of the alfalfa plastocyanin promoter. The authors also proved that the full hemagglutinin alone could assemble and form VLPs from plant cells. These VLPs are budded from the plant plasma membrane (D'Aoust *et al*., 2008). Full hemagglutinin of the virus strain NIBRG-14 was over-expressed using the magnICON (Icon Genetics, Halle, Germany) provector system, however an evidence of VLP structure was not shown (Kalthoff *et al*., 2010). Co-expression of at least hemagglutinin and matrix protein 1 (M1) was required to form VLPs in insect cells (Sf9 cells) (Galarza *et al.*, 2005). In mammalian cells, expression of hemagglutinin alone was sufficient to drive VLP formation, however the supplement of exogenous bacterial neuraminidase into the culture medium was required to release newly formed VLPs into the culture medium (Chen *et al.*, 2007). Chen and co-workers explained that

the neuraminidase activity was necessary to remove sialic acids from host glycoproteins. This action prevents aggregation of newly formed viruses on mammalian cell surface. In plant cells, plant glycoproteins do not contain sialic acids, therefore hemagglutinin-based VLPs could be formed and budded from plasmamembrane (D'Aoust *et al*., 2008).

The ectodomain of hemagglutinins (^{Nt}H5 and ^{Nt}H5-ELP) were successfully expressed in plant cells by use of a transient expression system (Fig. III-6A). However, these proteins were expressed as solely monomeric proteins (Fig. III-23A, 23B and 24), while hemagglutinin of influenza virions is a homotrimeric protein (Gamblin and Skehel, 2010). Therefore, an isoleucine zipper trimerization motif (GCN4-pII) (Harbury *et al*., 1994; Harbury *et al*., 1993) was fused to the ectodomain hemagglutinin at the C-terminus (Fig. III-3C). Similarly, the ectodomain of neuraminidase ELP fusion protein was a mixture of monomeric, dimeric and tetrameric proteins (Fig. III-23C). To express this neuraminidase as a tetrameric protein, a tetramerization motif (GCN4-pLI) (Harbury *et al*., 1993) was N-terminally fused to neuraminidase (Fig. III-3C). In this study, ectodomains of hemagglutinin and neuraminidase alone as well as their ELP fusions were expressed in plant cells under control of the ubiquitous promoter (CaMV 35S) and seed specific USP promoter. With constructs under control of the seed specific USP promoter, co-transformation with Agrobacteria harboring the vector for expression of the transcription factor FUS3 was required (Schallau, 2008). This allows to test expression of influenza antigens under control of the USP promoter without the need of transgenic seeds. Most recombinant proteins except monomeric and tetrameric neuraminidase were dectected 4 days after agroinfiltration (Fig. III-6 and 7). Interestingly, trimerization and tetramerization fusions to the C and N-termini of hemagglutinin and neuraminidase, respectively did not affect expression of these proteins (Fig. III-6 and 7).

Transient expression system using *N. benthamiana* is not only used to test ability of expression of target proteins but also to provide leaf materials for protein production. 4 days after agroinfiltration, trimeric hemagglutinin proteins (^{Nb}(H5pII)3 and ^{Nb}(H5pII-ELP)3) were purified immediately either by affinity chromatography (Fig. III-13) or mITC (Fig. III-18D and 21C). At our conditions, the first purified lot of research candidate vaccines (vaccines enough for preclinal examination about milligrams) would be available after only 3-4 weeks. With this vaccine production timeline, a strain-specific pandemic vaccine could be produced promisingly in a timely fashion by an alternative plant substrate. Using transient expression in plants could supply influenza pandemic vaccines in real time of pandemic (Fig. IV-1, Palache and Krause, 2008). The combination between speed of transient expression system and simpleness, easy scalability, less time consume and high efficiency of mITC purification method could enhance

the value of the plant-made candidate vaccines in a context of influenza pandemic. D'Aoust and co-workers previously reported that only 3 weeks were required to obtain the research grade vaccines against the novel A/H1N1/2009 strain (D'Aoust *et al*., 2010). Additionally, the transient expression system is an attractive way that was used to produce seasonal influenza vaccine candidates (Shoji *et al*., 2008; Mett *et al*., 2008). A rapid response in influenza vaccine production is the most advantage of plant-based transient expression system in comparison to egg-based technology. Manufacturing embryonated egg-driven vaccines evolves in multiple steps. These include the identification, preparation and verification of the vaccine strain that adapted to grow well in chicken eggs; the virus growth conditions of vaccine virus strain is optimized in eggs; manufacturing and purification of vaccines, etc. In the best case scenario, the full process takes 5-6 months to make the first pandemic vaccine lot available for distribution and use after a new pandemic vaccine strain is identified (WHO, 2009; Wright, 2008).

After the plant constructs under control of both promoters were successfully expressed transiently, they were stably transformed into *N. tabacum*. Western blot analyses showed that monomeric and trimeric hemagglutinins (^{Nt}H5 and ^{Nt}(H5pII)3) and ELP fusion proteins (^{Nt}H5-ELP and ^{Nt}(H5pII-ELP)3, ^{Nt}HA1-ELP, ^{Nt}HA2-ELP, ^{Nt}N1-ELP and ^{Nt}(pLI-N1-ELP)4) were successfully expressed in transgenic plant leaves (Fig. III-8 and 9). We found that ^{Nt}HA1-ELP and ^{Nt}HA2-ELP were more sensitive to plant proteases than full hemagglutinins (^{Nt}H5 and ^{Nt}H5-ELP) and trimeric hemagglutinins (Fig. III-8, 9, 16 and 17). In the native structure of hemagglutinin, there are disulfide bonds formed by Cys residues between subunit 1 and 2 (Khurana *et al*., 2011; Segal *et al*., 1992). It can be assumed that when subunits of hemagglutinin were expressed separately, hemagglutinin subunits were folded improperly due to lacking disulfide bonds. Therefore, they were highly susceptible to plant proteases. In contrast, ectodomain hemagglutinin and trimeric were not susceptible to plant proteases, especially oligomeric hemagglutinin is known as no trypsin susceptible protein (Wang *et al*., 2006). ^{Nt}HA1-ELP, ^{Nt}HA1, ^{Nt}H5-ELP, ^{Nt}H5 and ^{Nt}N1-ELP were also successfully expressed in transgenic seeds (Fig. III-11). Furthermore, homozygous lines expressing ^{Nt}H5, ^{Nt}H5-ELP, ^{Nt}HA1-ELP and ^{Nt}N1-ELP were selected (Table III-6). The stable transgenic plants could provide massive material for hemagglutinin purification.

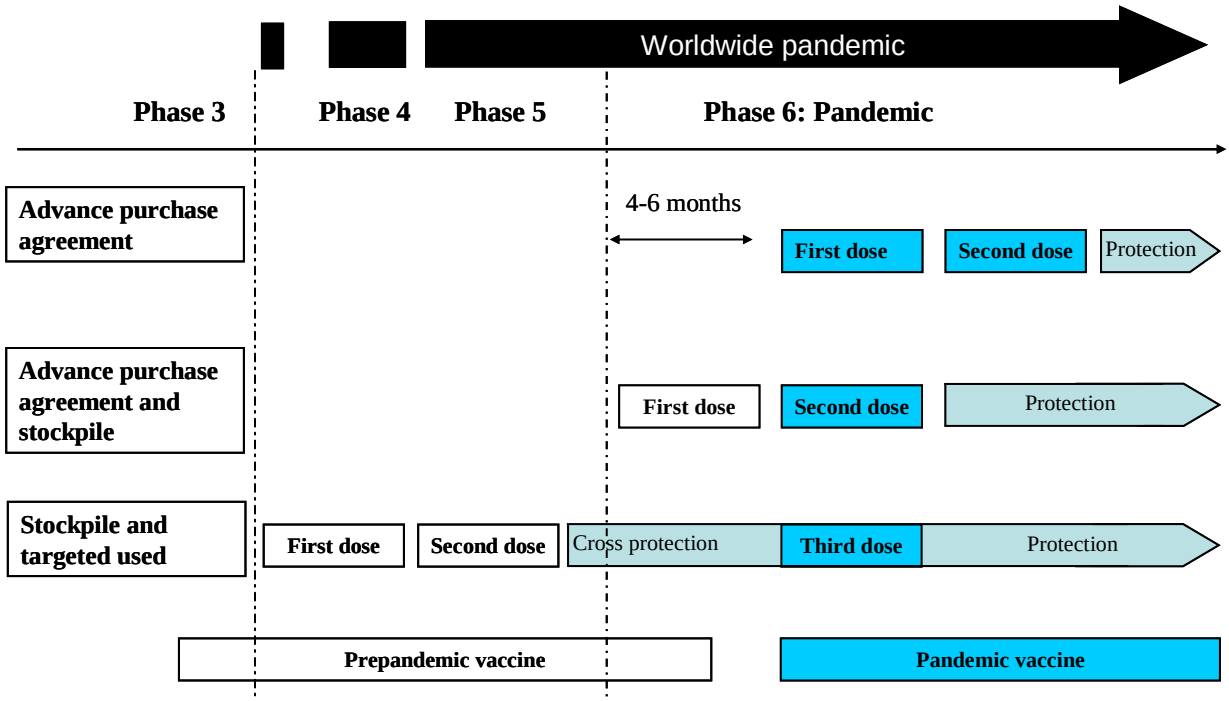

Figure IV-1. Potential vaccination strategies to protect against pandemic influenza showing administration timing based on the WHO pandemic-alert levels (Palache and Krause, 2009).

A question could be asked whether influenza vaccines from stably transformed plants could be useful for vaccination against influenza H5N1 viruses due to time-consuming production. In fact, most of the human population is naïve to H5N1 viruses, they do not possess immunity against this HP virus strain. The traditional process of influenza vaccine production in ECE is lengthy and pandemic influenza vaccines are not available in time to limit the spread of the novel influenza viruses. As a consequence, prepandemic vaccination is produced and licensed to prime human population (Palache and Krause, 2009). Prepandemic dose for population priming could be one or two (Fig. IV-1; Palache and Krause, 2009; Rappuoli and Giudice, 2008). Population priming by prepandemic vaccines provides full protection against homologous strains (after 2 doses) and cross-protective antibodies and memory B and T cells. When a pandemic is declared, available pandemic vaccines are used as a single dose. This strategy is now proven with humans and the only option that could prevent and control the risk of a pandemic (Rappuoli and Giudice, 2008). Stephenson and co-workers showed that this strategy could induce very fast cross-protective antibodies with only one booster dose. The primed subjects who had been administrated two or three doses of MF59 adjuvanted-A/duck/Singapore/97(H5N3) vaccines at least 6 years earlier were vaccinated with 7.5 µg of MF59-adjuvanted vaccine (clade 1, NIBRG-14 (H5N1)). 7 days after the 1st boost dose, at least 80% of MF59-primed subjects had serum HI titers of at least 40 against four different H5N1 clades (Stephenson *et al*., 2008).

In this study, the plant-produced trimeric hemagglutinins could induce neutralizing antibodies against homologous strain (90%) after two doses of abISCO adjuvanted vaccine candidates (Fig. III-37). Furthermore, these vaccine candidates were able to induce cross-reactive antibodies against the heterologous strain (clade 2.2.2) (Fig. III-39, 40 and 41). Based on these studies, we hyphothesize that the stably tranformed plant-produced trimeric hemagglutinins could be used as an alternative prepandemic influenza vaccines against H5N1 viruses. Influenza vaccines produced from stable transformation could be used in phase 4 and 5 of pandemic to prime population (Fig. IV-1).

IV.2. ELP enhancing accumulation of recombinant proteins in plants

Low accumulation level of recombinant proteins in plant cells is one of major challenges to limit the economic production of plant-made proteins (Conley *et al.*, 2011; Joensuu *et al.*, 2010). To circumvent this limitation, numerous strategies have been developed to enhance significantly the expression level of recombinant proteins in plant cells. These include fusion proteins (Joensuu *et al*., 2009; Mainieri *et al*., 2004, Floss *et al*., 2009); codon optimization (Joensuu *et al*., 2009) and using viral expression systems (Marillonnet *et al.*, 2004). Among these, the fusion protein strategy has been widely exploited since protein fusion partners not only result in increasing expression level of target proteins but also may assist subsequent purifications (Conley *et al*., 2011). Protein partners fused to target proteins could be enzymes: β-glucuronidase (Dus Santos *et al.*, 2002; Gil *et al.*, 2001), lichenase (Mett *et al*., 2008); cholera toxin B subunit (Arakawa *et al.*, 2001; Molina *et al.*, 2004); human Fc antibody fragment (Obregon *et al.*, 2006; Spitsin *et al.*, 2009) and protein body-inducing fusions (for review see Conley *et al*., 2011) including Zera/Zeosin (Mainieri *et al.*, 2004), ELP (Floss *et al*., 2009; Joensuu *et al*., 2009; Scheller *et al*., 2006) and hydrophobin (Joensuu *et al.*, 2010).

In this study, 100xELP was C-terminally fused to hemagglutinin and neuraminidase. The fusion proteins were kept in the ER by the KDEL ER localization signal peptide at the C-terminus of recombinant proteins. As shown in Fig. III-6 and 7, it is clear that the C-terminal ELP fusion resulted in increasing expression levels of all ELP fused proteins including monomeric, oligomeric hemagglutinins and neuraminidases under control of both the CaMV 35 promoter (Fig. III-6) and seed specific USP promoter (Fig. III-7). No immunogical signals were detected when neuraminidase was expressed alone, while neuraminidase ELP fusions were clearly detectable (Fig. III-6B and 7B). Patel and co-workers already showed that fusion with the 11.3 kDa ELP (27xELP) resulted in enhancing accumulation of human interleukin-10 (IL10) and murine interleukin-4 (IL4) approximately 15 and 19-fold, respectively.

Additionally, transient expression of MaSp2 spider silk proteins was approximately 100-fold higher than MaSp2 alone. However, the concentrations of recombinant IL10 and IL4 without ELP fusion were extremely low approximately 0.05% in both cases (Patel *et al*., 2007). Our results are consistent with earlier observations which showed that expression levels of ELP fused light chains and heavy chains of the anti-HIV antibodies (2F5 and 2G12) were higher than those of light chains and heavy chains of the anti-HIV antibodies without ELP fusions (Floss, 2008). These results are a direct evidence to prove that increasing accumulation of recombinant proteins is caused by a positive effect of ELP instead of position effects of transgenes because transient assays were conducted.

Increasing expression levels of recombinant proteins caused by ELP fusion was also observed in transgenic plants. In this study, fusing the 100xELP tag to the C-terminal hemagglutinins and neuraminidases enhanced significantly the expression of these proteins in both transgenic leaves and seeds (Fig. III-10 and 11). Expression levels of ^{Nt}HA1-ELP, ^{Nt}H5-ELP, ^{Nt}N1-ELP were 30, 12.5 and 50 fold higher than those of ^{Nt}HA1, ^{Nt}H5 and ^{Nt}N1, respectively. If ^{Nt}HA1-ELP and ^{Nt}N1-ELP had very high increase fold numbers (30 and 50-fold, respectively), it is because ^{Nt}HA1 and ^{Nt}N1 were expressed in transgenic plants with very low concentrations approximately 0.01 and 0.004% of TSP, respectively. Accumulation of ^{Nt}(H5pII-ELP)3 trimeric hemagglutinin was 2-fold higher than that of ^{Nt}H5-ELP monomeric hemagglutinin with levels 0.5 and 1% of TSP, respectively. Expression level of ^{Nt}N1-ELP was consistent with that of ^{Nt}(pLI-N1-ELP)4 tetrameric neuraminidase approximately 0.2%. In general, increasing accumulation of ELP fusion influenza proteins was in accordance with the literature and demonstrated that ELP fusion resulted in enhancing the production yield of target proteins by 2-100-fold. In the vegetative organs, Patel and co-workers previously reported that ELP fusion IL4, IL10 and MaSp2 proteins levels were 85, 90 and 60-fold higher than IL4, IL10 and MaSp2 alone, respectively. Without ELP fusion, IL4, IL10 and MaSp2 proteins were poorly detectable. Similarly, Scheller and co-workers presented the expression of scFvs fused to 100xELP in transgenic tobacco seeds. This strategy resulted in a 40 fold increase in scFv accumulation up to 25% of total soluble protein (Scheller *et al*., 2006).

Increasing accumulation of recombinant proteins caused by ELP was only obtained when ELP tag was fused to the C-terminus of target proteins and recombinant proteins were located in the ER compartment. (Conley *et al*., 2009b). Conley and co-workers showed that the ELP tag had negligible effect on expression level of GFP in the cytoplasm and apoplast, while it significantly reduced the concentration of GFP in the chloroplast (Conley *et al*., 2009b). Increasing expression levels of ELP fusion proteins could be caused by several reasons. ELP

fusion proteins were not susceptible to host proteases (Zhang *et al.*, 1996) and hydrolysis (Raucher and Chilkoti, 2001), therefore reducing their degradation. The ELP tag increased the solubility of ELP fusion proteins by preventing irreversible aggregation and denaturation at high protein concentrations (Trabbic-Carlson *et al*., 2004). Recent studies showed that the ER targeted ELP fusions were accumulated into a novel protein body (PB) in leaves (Conley *et al*., 2009b) and seeds of tobacco (Floss *et al*., 2009). The formation of PBs are thought to enhance the accumulation of ELP fusion proteins because the recombinant proteins in PBs are protected from proteolytic degradation and excluded from normal physiological turnover, thus minimizing unnecessary stress to the host cell (Conley *et al*., 2009b).

In summary, ELP fusions enhanced significantly the expression levels of plant-made recombinant proteins. This results in higher concentration of the target proteins in the initial aqueous extraction. Thus, subsequent purification is more feasible because successful purification depends heavily on inducing a high concentration of the target molecule in the plant material (Floss and Conrad, 2011).

IV.3. ELP-based purification of vegetable-expressed antigens

The extraction, recovery and purification of subunit vaccines that normally involves chromatography and associated labor and capital equipment represent over 80% of the overall production cost. These costs are independent on the primary production system, but *in planta* systems are less demanding than *in vitro* ones with respect to the upstream components, as they avoid the need for fermentation equipment and culture media (Phan and Conrad, 2011; Yusibov and Rabindran, 2008). As a result, the per gram cost of therapeutic proteins produced by mammalian cells is substantially higher than that from plants (Conley *et al.*, 2009b). The major technical bottleneck associated with *in planta* production lies in the down-stream processing procedure (Conley *et al.*, 2009a).

The fusion of ELP tag to proteins is known to enhance the accumulation of transgenic proteins *in planta* (for review see Conley *et al*., 2011; Floss *et al*., 2010b). The ELP tag has the further benefit of simplifying the subsequent purification *via* ITC, a method based on the inherent reversibility of ELPylation (section I.3; for review see Floss *et al*., 2010b). ELP fusion proteins could be purified by the cITC method which is based on the precipitation of ELPylated proteins by a combination of salting, heating, centrifugation and resolubilization in the absence of salt at a low temperature (Meyer and Chilkoti, 1999). This method has been successfully used to purify vaccines (Floss *et al*., 2010a), complete immunoglobulins (Floss *et al*., 2008; Floss *et al*., 2009), antibody fragments (Conrad *et al*., 2011; Joensuu *et al*., 2009)

and several other proteins from plant materials (Conley *et al*., 2011; for review see Floss *et al*., 2010b). In this study, although hemagglutinin and neuraminidase ELP fusion proteins were also successfully purified by cITC, ELPylated proteins could be isolated only at low concentration and contaminated a lot of impurities. Recovery rates of these proteins were low and variable (Table III-8). Moreover, target proteins were completely cleaved by plant proteases during cITC purification process (Pc, Fig. III-17A and 18A). In general, cITC was not suitable to purify ELP fused influenza hemagglutinin and neuraminidase proteins from plants.

There is another method that has been used to isolate the precipitate of ELP fusion proteins by the use of microfiltration (Ge *et al*., 2006). This method relies on the ability to retain the precipitation of ELP fusion proteins on the surface of a membrane, termed membrane-based ITC (mITC) becauce of micrometer size aggregates of the ELP fusion proteins (Ge *et al*., 2006; Meyer and Chikoti, 2002). The protocol was optimized to purify ELP fusion proteins from bacterial cell lysate (Christensen *et al*., 2007; Ge *et al*., 2006). Therefore, in order to purify ELP fusion proteins from transgenic leaves, this protocol had to be further optimized. High speed centrifugation (75,600 *g*) and prefilter (with a 0.22 µm membrane) at 4 °C were required to make the plant extract clear enough for mITC (section III.5.3.2). The improved mITC was applied to purify successfully three different ELP fusion proteins including ^{Nt}HA1-ELP, ^{Nt}H5-ELP and ^{Nt}N1-ELP from transgenic leaves with highly and constantly efficient recovery approximately 95, 95 and 92.5-94%, respectively (Phan and Conrad, 2011). These results were consistent with the previous observation by Ge and co-workers who reported that Trx-ELP was recovered from bacterial cell lysate up to 95% by mITC (Ge *et al*., 2006). Importantly, mITC purified proteins had high purity degrees ranging from 81 to 89% (Table III-8). These purity degrees were in accordance with those of influenza hemagglutinins that were purified by affinity chromatography (D'Aoust *et al*., 2008; Mett *et al*., 2008). Moreover, ELP fusion protein was protected from plant proteases by using improved mITC method because plant proteases were passed through a membrane during purification process. Improved mITC was also adapted to purify ^{Nb}(H5pII-ELP)3 from transiently transformed *N. benthamiana* leaves resulting in 97% of efficient recovery. Success in purifying hemagglutinin ELP fusion from transient leaf materials by mITC is a very important step to speed up influenza production vaccines because mITC is simple and easy to scale up for industrial applications. The advantage of mITC is that the precipitation of ELP fusion proteins are retained on the surface of a membrane, therefore purification process is less time-consuming and ELP fusion proteins are subsequently easy to elute from membrane.

The results of this study showed that it is possible to use an ELP fusion technology for purification of plant-made pharmaceutical proteins. The ELP fusion influenza antigens were highly enriched by mITC. This method is simple, highly efficient, scalable and less time-consuming, especially to avoid degradation of recombinant influenza antigens caused by plant proteases. However, for human application, further purification steps (gel chromatography) are necessary to have sufficient purity of target proteins. The enhanced concentration of the influenza antigen ELP fusions and a scalable and cheaper purification method could reduce significantly total cost of influenza subunit vaccines.

IV.4. Functionality of plant-produced influenza antigens

In this study, the hemagglutinin and neuraminidase proteins expressed in plant expression system were highly soluble even at relatively high concentration (Fig. III-13, 14 and 21). These results are similar with previous studies in which these proteins were expressed in plants (D'Aoust *et al*., 2008; Kalthoff *et al*., 2010; Mett *et al*., 2008; Shoji *et al*., 2009a, Shoji *et al*., 2009b; Spitsin *et al*., 2009), insect cells (Deroo *et al*., 1996 ; Weldon *et al*., 2010) and mammalian cells (Bosch *et al.*, 2010; Cornelissen *et al.*, 2010; Du *et al*., 2011). In contrast, hemagglutinins expressed in *E. coli* were directed to the insoluble fraction (inclusion bodies-IBs). These IBs must be refolded *in vitro* under controlled redox conditions (Biesova *et al.*, 2009; Khurana *et al.*, 2010; Khurana *et al.*, 2011).

We found that ectodomain hemagglutinins (both ^{Nt}H5-ELP and ^{Nt}H5) expressed in plants were solely present as monomers which were confirmed by cross-linking reaction and a blue native PAGE (Fig. III-23A, 23B and 24). As a result, these proteins did not agglutinate chicken RBCs (Fig. III-26 and Table III-11). Although ^{Nt}HA1-ELP had the ability to form trimers (Fig. III-23D), this protein did not show agglutination of chicken RBCs (Table III-11). The reason could be that the proportion of trimers was markedly lower than that of monomer. Similarly, full or partial (1-480 aa) hemagglutinin ectodomain was expressed as monomeric proteins in *E. coli* (Khurana *et al*., 2010) and insect cell (Weldon *et al*., 2010), respectively. Khurana and co-workers previously reported that in the *E. coli* expression system, hemagglutinin global domain (1-320 and 1-330 but not 1-480) could form oligomeric state and the oligomerization signal was the N-terminus $I_3C_4I_5$ residues (Khurana *et al*., 2011). When a trimeric motif GCN4pII was introduced at the C-terminal hemagglutinin ectodomain, the trimeric hemagglutinins (^{Nb}(H5pII)3 and ^{Nb}(H5pII-ELP)3) were stably formed and confirmed by cross-linking reactions and a blue native PAGE (Fig. III-23A-B and 24). Both approaches provide the advantage to determine oligomeric state of hemagglutinins without using complex

gel chromatography technique. It is clearly visible that both trimeric hemagglutinin ELP fusion protein ^{Nb}(H5pII-ELP)3 and its trimeric counterpart ^{Nb}(H5pII)3 could agglutinate chicken RBCs (Fig. III-26 and Table III-11). Similar to this study, the multimeric states of *E. coli*, mammalian and insect cell-based hemagglutinin showed hemagglutination compared to their monomeric counterparts (Cornelissen *et al*., 2010; Du *et al*., 2011; Khurana *et al*., 2010; Khurana *et al*., 2011; Weldon *et al*., 2010). To express ectodomain of glycoproteins similar in structure and function of native viral glycoproteins, oligomerization motives have been used in recent reports. These motives could be the tetrameric GCN4-pLI motif (Bosch *et al*., 2010; Harbury *et al*., 1993), trimeric motives including the trimeric fibritin foldon motif (Du *et al*., 2011; Sissoeff *et al*., 2005) and trimeric GCN4-pII motif (Harbury *et al*., 1993; Weldon *et al*., 2010).

Although hemagglutinin and neuraminidase are the targets of antibodies that block infection (Gamblin and Skehel, 2010), only hemagglutinin content in influenza vaccines are standardized because this protein is the primary target of neutralizing antibodies and an much more abundant surface protein in comparison to neuraminidase (Sandbulte *et al*., 2007). Recent reports showed that immunization with neuraminidase protein could induce cross-reactive antibodies to protect partially mice from lethal H5N1 (Sandbulte *et al*., 2007) or could strongly enhance the hemagglutinin-specific responses (Bosch *et al*., 2010; Mett *et al*., 2008). In this study, ELP fusion neuraminidase was expressed and purified by mITC from transgenic plants. This protein is a mixture of monomeric, dimeric and tetrameric proteins. Oligomeric state of neuraminidase proteins was determined by cross-linking reaction (Fig. III-23C). Monomeric and dimeric proteins were abundant, tetrameric protein was minor. In order to express this protein as a tetrameric protein, the tetramerization motif (Harbury *et al*., 1993) was fused to the N-terminal neuraminidase (Fig. III-3C). Biological activity of ^{Nb}(pLI-N1-ELP)4 tetrameric neuraminidase was not detectable, although it was successfully purified from transiently transformed *N. benthamiana* leaves (Pm, Fig.III-18C and Fig.VII-2A). In contrast, ^{Nt}(pLI-N1-ELP)4, which was purified from stably transformed plants, was enzymatically active, although the same expression construct was used for both transient and stable transformation. This can be due to lack of post-translational modifications or incomplete folding of ^{Nb}(pLI-N1-ELP)4 that was over-expressed in transiently transformed *N. benthamiana* leaves. The ^{Nt}(pLI-N1-ELP)4 tetrameric neuraminidase purified from the line 32 of the stable transformation neuraminidase exhibited a specific activity of 314 U/mg. This value is in agreement with the previous report with specific viral activities: rgH1N1 = 294 U/mg, rgH5N1 = 378 U/mg and rgH1N3 = 356 U/mg (Yongkiettrakul *et al*., 2009). ^{Nt}N1-ELP had a specific activity of 33.6

U/mg. It can be seen that the specific activity of ^{Nt}(pLI-N1-ELP)4 is 9-fold higher than that of ^{Nt}N1-ELP. This difference could come from differently oligomeric state of two proteins. ^{Nt}(pLI-N1-ELP)4 is an exclusively tetrameric protein, while ^{Nt}N1-ELP is mainly monomeric, dimeric proteins (Fig. III-23C). Deroo and co-workers showed that enzymatic activity was only associated with the tetrameric form of influenza neuraminidase (Deroo *et al*., 1996). Based on these analyses, ^{Nt}(pLI-N1-ELP)4 tetrameric neuraminidase is similar in structure and function to viral neuraminidase.

Taken together, functionalities of hemagglutinin and neuraminidase associate strongly with trimeric and tetrameric structures of these proteins, respectively. As already reported (Floss *et al*., 2010b), ELP fusion technology likely remains functionalities of recombinant proteins.

IV.5. Trimeric but not monomeric hemagglutinins induce neutralizing antibodies

Although chicken vaccinated with a single dose of ^{Nt}H5-ELP monomer and ^{Nt}HA1-ELP showed immunological responses to the ^{Nt}H5-ELP and ^{Nt}HA1-ELP (Fig. III-31), these monomeric hemagglutinins failed to induce neutralizing antibodies against homologous VLPs and heterologous strain. Therefore all chickens died after virus challenge. HI results showed that neutralizing antibodies were not induced by monomeric hemagglutinins after single immunization of chickens. These data are in agreement with previous reports using the virus A/Aichi/2/68(H3N2) soluble hemagglutinin (Weldon *et al*., 2010), the virus A/Victoria/3/75(H3N2) soluble hemagglutinin (Vanlandschoot *et al*., 1996). These reports showed that two immunizations of monomeric soluble hemagglutinin failed to induce neutralizing antibodies. As a result, mice vaccinated with these monomeric hemagglutinins of A/Victoria/3/75(H3N2) and A/Aichi/2/68(H3N2) strain were not protected against homologous virus challenge (Vanlandschoot *et al*., 1996) or partially protected (33%) against homologous strain (Weldon *et al*., 2010), respectively. Spitsin and co-workers also reported that the plant-made HA1 variants (both fusion and no fusion with Fc fragment) from A/Vietnam/1203/2004(H5N1) did not induce neutralizing antibodies (Spitsin *et al*., 2009). Monomeric hemagglutinins (^{Nt}H5 and ^{Nt}H5-ELP) also did not induce neutralizing antibodies against homologous VLPs after the third immunization (Fig. III-34A and 34B) or poorly induced these antibodies after the fourth immunization (Fig. III-34C and 34D). In contrast to this study, plant-made extracellular hemagglutinins from A/Bar-headed Goose/Qinghai/1A/05(H5N1) (Shoji *et al*., 2009b) and A/Indenesia/05/05(H5N1) (Shoji *et al*., 2009a) could induce strong HI titers after the third immunization. Moreover, vaccinated ferrets

were protected from homologous A/Indonesia/05/05(H5N1) virus (Shoji *et al*., 2009a). The difference between our study and Shoji's report is the adjuvant. Freund's adjuvant was used in our study, whereas Quil A adjuvant was utilized in Shoji's researches (Shoji *et al*., 2009a; Shoji *et al*., 2009b).

The ^{Nb}(H5pII)3 and ^{Nb}(H5pII-ELP)3 trimeric hemagglutinins elicit higher levels of total IgG than ^{Nt}H5 and ^{Nt}H5-ELP monomeric hemagglutinins, respectively (Fig. III-32). Moreover, trimer-induced antibodies have stronger binding affinities than antibodies induced by monomeric hemagglutinins (Fig. III-33). Similarly, Weldon and co-workers previously reported that mice vaccinated with trimeric hemagglutinin induced approximately 15-fold higher serum IgG titers than the monomeric hemagglutinin immunized mice (Weldon *et al*., 2010).

Unless otherwise stated, neutralizing antibodies against homologous VLPs were induced after two immunizations of abISCO-adjuvanted trimeric hemagglutinins and HI titers were significantly increased after the third immunization (Fig. III-37). These findings are consistent with the previous observations which showed that stabilized oligomeric and trimeric hemagglutinins could induce significantly neutralizing antibodies (Bosch *et al*., 2010; Cornelissen *et al*., 2010; Wei *et al*., 2008; Weldon *et al*., 2010). Interestingly, the previous reports showed that mice vaccinated with soluble trimeric hemagglutinin of the A/Aichi/2/68(H3N2) strain were partially (5/6) or fully protected against homologous strain after a single dose and two doses, respectively (Weldon *et al*., 2010). Cornelissen and co-workers also demonstrated that two immunizations of 10 µg of soluble trimeric hemagglutinin from the A/Vietnam/1203/2004(H5N1) strain were enough to fully protect vaccinated chickens against the homologous strain (Cornelissen *et al*., 2010). Results from this study and others demonstrate that oligomeric and trimeric hemagglutinins have the ability to efficiently elicit neutralizing antibodies in vaccinated animals, whereas monomeric hemagglutinins do not have that capacity. This difference could be due to different epitopes which are present in the trimeric and monomeric forms. The trimeric hemagglutinin possesses epitopes that could induce neutralizing antibodies, while the monomeric hemagglutinins do not exhibit these epitopes, therefore neutralizing antibodies are not induced. Sandwich ELISA data from Weldon and co-workers showed that the monoclonal antibody HC67 that is specific for the trimeric hemagglutinin of virus A/Aichi/2/68(H3N2) bound recombinant trimeric hemagglutinin of this strain with a higher affinity compared to the recombinant monomeric hemagglutinin. Similarly, the monoclonal antibody LC89 that is specific for the monomeric hemagglutinin of virus A/Aichi/2/68(H3N2) bound recombinant monomeric hemagglutinin of

this strain with a higher affinity than the recombinant trimeric hemagglutinin (Weldon *et al*., 2010).

In this study, homologous VLPs rather than H5N1 viruses used in HI assays provide us ability to measure neutralizing antibodies against homologous the A/Hatay/2004(H5N1) strain. Although VLPs must be purified, full process working with VLPs can be performed under biosafety level 1&2 conditions because of non infectious VLPs. Moreover, VLPs are useful to assess neutralizing antibodies against the completely homologous A/Hatay/2004(H5N1) strain, while this viral strain is not available. Cornelissen and co-workers reported that the trimeric hemagglutinin could be used for HI assay. This approach demonstrated the reliability of the assay and gave essentially similar result in comparison to using the inactivated H5N1 virus (Cornelissen *et al*., 2010).

Furthermore, the plant-made trimeric hemagglutinins also elicited high cross-reactive neutralizing antibodies against heterologous strain rg A/swan/Germany/R65/2006(H5N1) that have 96% hemagglutinin amino acid sequence similarity with the hemagglutinin of A/Hatay/2004(H5N1) strain (Table VII-1). This is in agreement with recent reports which showed that cross-reactive neutralizing antibodies were induced by influenza vaccine formulated with novel adjuvants such as oil-in-water including AS03 (Leroux-Roel *et al*., 2007), MF59 (Podda, 2001), AF03 (Levie *et al*., 2008) and Matrix M^{TM} (Cox *et al*., 2011). Cross-reactive antibody responses induced by candidate vaccines are a very important property to combat antigenic diversity of H5N1 strains.

In this study, we found that both adjuvants (Freund and ISCOM-based Matrix M^{TM}) formulated with trimeric hemagglutinins could elicit neutralizing antibodies against homologous VLPs. Moreover, ISCOM-based Matrix M^{TM} adjuvant (abISCO adjuvant) was able to elicit immune responses with significantly higher titers than Freund's adjuvant (Fig. III-36). As a result, trimeric hemagglutinins formulated with Matrix M^{TM} adjuvant induced higher neutralizing antibodies against both homologous and heterologous strains (Fig. III-38 and 41). Previously published studies showed that the Matrix M^{TM} adjuvant is well tolerated and enhanced antibody responses. This adjuvant allows dose reduction to 1.5 µg antigen (Cox *et al*., 2011). The difference of immune responses could be due to different activities of the two adjuvants. Freund's adjuvant is a water-in-oil adjuvant that has adjuvant activity through depot effect. Freund formulated antigens are slowly released from injection site leading to induction of APC maturation and improvement of presentation of antigen to APCs (Atma and Keitel, 2009). In contrast to water-in-oil, the ISCOM or ISCOM-based Matrix M^{TM} adjuvant-antigen complexes have a particulate nature. They are rapidly removed from injection site to the

draining lymph nodes and are early uptaken by APCs such as dendritic cells (DCs) or macrophages by endocytosis (Morein and Bengtsson, 1998). This results likely in enhanced presentation of antigens (Sjolander *et al.*, 1998). As a result, CD8-restricted cytotoxic T lymphocytes (CTL) are induced (Morein and Bengtsson, 1998; Takahashi *et al.*, 1990). This immune response is very important to clear viral infection (Villacres *et al.*, 1998). Moreover, this adjuvant can induce balanced both Th1 and Th2 lymphocytes (Madhun *et al.*, 2009). Cytokines (IL2; IFNγ) that are produced by Th1 play an important role in promoting cell-mediated immunity that kills infected cells, while cytokines (IL4, IL-5 and IL-10) that are produced by Th2 play important role in humoral immunity by inducing proliferation and differentation of B cells, antibody class and subclass switching (Madhun *et al*., 2009). Briefly, ISCOMs adjuvant increased significantly both the humoral and cellular immune responses. In a phase I clinical study, a virosomal H5N1 vaccine formulated with the latest generation of ISCOM (Matrix M^{TM}) showed that the Matrix M^{TM} stimulates strong immune responses against both homologous and heterologous H5N1 strains. Additionally, this study showed an excellent safety profile of Matrix M^{TM} adjuvanted vaccine (Cox *et al*., 2011).

Taking immunization studies into consideration, the plant-made trimeric hemagglutinins are highly immunogenic and can elicit neutralizing antibodies against both homologous and heterologous strains. Vaccination with two doses of trimers are required to induce enough neutralizing antibodies. ISCOM-based Matrix M^{TM} adjuvant is a potential adjuvant to induce high immune responses.

IV.6. Low immunogenicity of the 100xELP tag

In this study, the feasibility of using the ELP-fused hemagglutinin as an influenza candidate vaccine was evaluated in animals. Furthermore, immunogenicity of the 100xELP tag was also assessed. Following immunization, immune responses are mainly targeted to hemagglutinin protein. No significant immune responses against 100xELP tag were observed in comparison to control group (Fig. III-42). These results are in agreement with the finding by Floss and co-workers who reported that the purified 100xELP tag did not induce proliferation of mouse and bovine lymphocytes. It also did not trigger expression of the maturation markers (MHCII, CD40, CD80 and CD86) on mouse and bovine dendritic cells that are key players in the immune responses (Floss *et al*., 2010a). Similarly, other studies indicated that ELPs were low immunogenic, but biodegradable and biocompatible when they were used as biomaterials for cartilage repair (Hrabchak *et al.*, 2010; McHale *et al.*, 2005; Ong *et al.*, 2006). These properties of ELPs are understandable because ELPs are artificial repetitive biopolymers of the

pentapeptide motif VPGXG that is present in the hydrophobic domain of tropoelastin across species (Floss *et al.*, 2010b; Gray *et al.*, 1973; Vrhovski and Weiss, 1998).

In summary, this study demonstated that the ELP fusion tag has low immunogenicity and no impact on immunogenicity of influenza hemagglutinin. Hence, it is not necessary to remove the 100xELP tag from the plant-made influenza antigens.

Conclusion and outlooks

In this study, influenza monomeric, multimeric hemagglutinin and neuraminidase proteins were expressed in both transiently transformed *N. benthamiana* and stably transformed *N. tabacum* as ELP fusion proteins. The 100xELP fusion partner significantly enhanced expression levels of these proteins in the tobacco leaves and seeds. A membrane-based inverse transition cycling (mITC) was successfully developed to recover ELPylated hemagglutinin and neuraminidase from plant material. Refinements of the mITC method are efficient, rapid, scalable and non-expensive. Purified multimers of hemagglutinin and neuraminidase ELP proteins and their counterparts showed structures and functionalities in similarity to hemagglutinin and neuraminidase of influenza virus. Freund and abISCO-adjuvanted trimeric hemagglutinins are able to induce neutralizing antibodies against both homologous VLPs (clade 1) and heterologous strain (clade 2.2.2). The 100xELP tag exhibits as a low immunogenic carrier protein and remains the immunogenicity of influenza hemagglutinin. Data in this study demonstrate that plant-made trimeric hemagglutinins present a promisingly alternative vaccine to fight against outbreaks of pandemic influenza viruses.

Scaling up experiments will be necessary to confirm that ELP-based purification approach using mITC will be able to circumvent the major bottleneck which at present inhibits the in *planta* production of antigens and antibodies. Further experiments will be required to determine whether recombinant plant-made trimeric hemagglutinin proteins and tetrameric neuraminidase can protect model animals from influenza HP H5N1 viruses. In addition, effects of ELP fusion on glycosylation patterns should be also monitored.

V. Summary

Currently, the worldwide emergence of the novel influenza A H5N1 with high mortality rates of 59% (332 deaths/566 human cases) has raised concern that this virus subtype could adapt to transmit effectively among humans and cause a next pandemic. Although influenza vaccines have been produced traditionally in ECE over 60 years and used efficiently as seasonal vaccines, the lengthy production process and limited vaccine production capacity of the egg-based technology do not facilitate a rapid response during a pandemic situation. For these reasons, many attempts are under way to develop rapid, efficient and reliable vaccine production technologies. Among these, developing recombinant influenza vaccines is a promising approach followed by influenza vaccine manufactures. Recently, plants have emerged as a potential means to produce recombinant proteins because they offer several advantages.

Hemagglutinin and neuraminidase (monomeric and oligomeric forms) from the highly pathogenic avian influenza (HPAI) A/Hatay/2004/(H5N1) strain were optimally expressed in tobacco plants in terms of their functionalities and protein yields by fusing with elastin-like polypeptides (ELPs) at the C-terminus. Recombinant proteins were produced successfully in both stable transgenic tobacco plants and in transiently transformed *Nicotiana benthamiana*. An enhancement of the accumulation of recombinant influenza hemagglutinin and neuraminidase in plants based on ELP-fusion technology was confirmed in both seeds and leaves of transgenic plants.

The ELP-fusion technology also provides a means for purification of ELP fusion proteins based on inverse transition cycling (ITC). Membrane-based ITC was developed and applied to successfully purify ELP fused hemagglutinin and neuraminidase proteins from leaf materials. This method is simple, highly efficient, scalable and less time-consuming. It is especially useful to avoid degradation of recombinant influenza antigens caused by plant proteases.

Characterization of purified influenza hemagglutinins and neuraminidases by the hemagglutination assay and enzymatic activity using 2’-(4-methylumbelliferyl)-α-D-N-acetylneuraminic acid as a substrate, respectively, showed that trimeric hemagglutinins were able to agglutinate chicken erythrocytes, while monomeric hemagglutinins were not able to do so. Similarly, tetrameric neuraminidase exhibited a 9-fold higher enzymatic activity than monomeric neuraminidase. ELP fusion does not affect functionalities of these proteins.

The immunogenicity of monomeric and trimeric hemagglutinins was evaluated in mice and the immunogenicity of monomeric hemagglutinins was evaluated in chicken. Monomeric

hemagglutinins failed to induce neutralizing antibodies, therefore chickens vaccinated with monomeric hemagglutinins were not protected from lethal HP H5N1 virus challenge. In contrast to monomers, trimeric hemagglutinins could induce high neutralizing antibody titers against homologous virus-like particles that were produced in transiently transformed *N. benthamiana* plants and against a heterologous inactivated virus strain. Low immunogenicity of the 100xELP tag was confirmed in this study by ELISA. Further work will focus on the application of these findings in extended chicken studies.

Zusammenfassung

Das weltweite Auftreten des neuen Influenza A Virus H5N1 mit hohen Mortalitätsraten von 59% (332 Todesfälle von 566 Fällen) lässt befürchten, dass dieser Virussubtyp sich anpasst und Menschen durch Menschen infiziert werden können. Das könnte zu einer neuen Pandemie führen. Obwohl Influenzavakzine seit 60 Jahren traditionell in embryonierten Hühnereiern produziert und effizient als saisonale Vakzine genutzt werden, erlauben der lange Produktionsprozess und die begrenzte Produktionskapazität der eierbasierten Technologie keine ausreichend schnelle Reaktion in einer Pandemiesituation. Aus diesen Gründen werden viele Versuche unternommen, um schnelle, effiziente und wirksame Vakzineproduktionstechnologien zu entwickeln. Aktuell werden Pflanzen aufgrund einer Reihe von Vorteilen durchaus als potentielles und wirksames Mittel zur Produktion rekombinanter Proteine angesehen.

Hämagglutinin und Neuraminidase (monomere und oligomere Formen) des hochpathogenen Vogelgrippevirusstammes (HPAI) A/Hatay/2004/(H5N1) wurden unter optimierten Bedingungen (Expressionshöhe und Funktionalität) in Tabakpflanzen exprimiert. Diese Proteine wurden C-terminal mit dem „Elastin-like-Peptide“ (ELP) fusioniert. Die rekombinanten Proteine wurden sowohl in stabil transformierten transgenen Tabakpflanzen als auch transient in *Nicotiana benthamiana* erfolgreich produziert. Sowohl für Samen als auch für Blätter wurde gezeigt, dass die ELP-Fusionstechnologie zu einer Erhöhung der Akkumulation von rekombinantem Hämagglutinin und rekombinanter Neuraminidase führt.

Die ELP-Fusionstechnologie ermöglicht auch die Reinigung ELPylierter Proteine durch „Inverse Transition Cycling“ (ITC). Eine ITC-Methode unter Nutzung von Membranfiltern zur Abtrennung der rekombinanten Proteine wurde entwickelt und erfolgreich zur Reinigung von Hämagglutinin-ELP und Neuraminidase-ELP aus Blättern genutzt. Diese Methode ist einfach, hocheffizient, skalierbar und erfordert wenig Zeitaufwand. Die Methode ist besonders nützlich, um den Abbau rekombinanter Influenzaantigene durch Pflanzenproteasen zu vermeiden.

Durch die Charakterisierung gereinigter Influenzahämagglutinine und Influenzaneuraminidasen durch den Hämagglutinationstest bzw. durch Enzymaktivitätstest mit 2’-(4-Methylumbelliferyl)-α-D-N-Acetylneuraminsäure wurde gezeigt, dass trimere Hämagglutinine im Gegensatz zu monomeren Hämagglutininen Hühnererythrozyten agglutinieren können. Tetramere Neuraminidase zeigte eine neunfach höhere Enzymaktivität als monomere Neuraminidase. Die Anwesenheit von ELP beeinflusste die Funktionen der beiden rekombinanten Proteine nicht.

Die Immunogenität der monomeren und trimeren Hämagglutinine wurde in Mäusen untersucht und die Immunogenität der monomeren Hämagglutinine zusätzlich in Hühnchen. Monomere Hämagglutinine induzierten keine neutralisierenden Antikörper. Deshalb zeigten Hühnchen, die mit monomeren Hämagglutininen immunisiert wurden, keinerlei Immunität nach einer letalen HP H5N1 Virusapplikation. Hohe Titer neutralisierender Antikörper gegen homologe Virus-artige Partikel (VLPs) und gegen heterologe inaktivierende Viren konnten durch trimere Hämagglutinine induziert werden, während durch Immunisierung mit monomeren Hämagglutininen keine neutralisierenden Antikörper erzeugt werden konnten. Die VLPs wurden durch transiente Expression in *N. benthamiana* –Pflanzen erzeugt. Durch ELISA-Tests wurde die niedrige Immunogenität von 100xELP gezeigt. Weitere Arbeiten werden sich auf die Anwendung dieser Befunde in umfangreichen Hühnerstudien konzentrieren.

VI. References

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VII. Appendix

VII.1 Amino acid sequences of recombinant proteins

H5-VLP (Molecular weight: 62178.48 Da)
LeB4 signal peptide: **MASKPFLSLLSLSLLLFTSTCLA**
GSDQICIGYHANNSTEQVDTIMEKNVTVTHAQDILEKTHNGKLCALDGVKPLILRDCSVAGWLLGNPMCDEFI
NVPEWSYIVEKANPVNDLCYPGDFNDYEELKHLLSRINHFEKIQIIPKSSWSSHEASLGVSSACPYQGKSSFFRN
VVWLIKKNSTYPTIKRSYNNTNQEDLLVLWGIHHPNDAAEQIKLYQNPTTYISVGTSTLNQRLVPRIATRSKVN
GQSGRMEFFWTILKPNDAINFESNGNFIAPEYAYKLVKKGDSTIMKSELEYGNCNTKCQTPMGAINSSMPFHNI
HPLTIGECPKYVKSNRLVLATGLRNSPQRERRGLFGAIAGFIEGGWQGMVDGWYGYHHSNEQGSGYAADKES
TQKAIDGVTNKVNSIIDKMNTQFEAVGREFNNLERRIENLNKKMEDGFLDVWTYNAELLVLMENERTLDFHD
SNVKNLYDKVRLQLRDNAKELGNGCFEFYHKCDNECMESVRSGTYDYPQYSEEARLKREEISGVKLESIGIYQI
LSIYSTVASSLALAIMVAGLSLWMCSNGSLQCRICI

HA0ER (Molecular weight: 65398.95 Da)
LeB4 signal peptide: **MASKPFLSLLSLSLLLFTSTCLA**
GSDQICIGYHANNSTEQVDTIMEKNVTVTHAQDILEKTHNGKLCALDGVKPLILRDCSVAGWLLGNPMCDEFI
NVPEWSYIVEKANPVNDLCYPGDFNDYEELKHLLSRINHFEKIQIIPKSSWSSHEASLGVSSACPYQGKSSFFRN
VVWLIKKNSTYPTIKRSYNNTNQEDLLVLWGIHHPNDAAEQIKLYQNPTTYISVGTSTLNQRLVPRIATRSKVN
GQSGRMEFFWTILKPNDAINFESNGNFIAPEYAYKLVKKGDSTIMKSELEYGNCNTKCQTPMGAINSSMPFHNI
HPLTIGECPKYVKSNRLVLATGLRNSPQRERRGLFGAIAGFIEGGWQGMVDGWYGYHHSNEQGSGYAADKES
TQKAIDGVTNKVNSIIDKMNTQFEAVGREFNNLERRIENLNKKMEDGFLDVWTYNAELLVLMENERTLDFHD
SNVKNLYDKVRLQLRDNAKELGNGCFEFYHKCDNECMESVRSGTYDYPQYSEEARLKREEISGVKLESIGIYQI
LSIYSTVASSLALAIMVAGLSLWMCSNGSLQCRICIAAAHHHHHHLAAEQKLISEEDLNGSKDEL

H5 (Molecular weight: 60865.45 Da)
LeB4 signal peptide: **MASKPFLSLLSLSLLLFTSTCLA**
GSDQICIGYHANNSTEQVDTIMEKNVTVTHAQDILEKTHNGKLCALDGVKPLILRDCSVAGWLLGNPMCDEFI
NVPEWSYIVEKANPVNDLCYPGDFNDYEELKHLLSRINHFEKIQIIPKSSWSSHEASLGVSSACPYQGKSSFFRN
VVWLIKKNSTYPTIKRSYNNTNQEDLLVLWGIHHPNDAAEQIKLYQNPTTYISVGTSTLNQRLVPRIATRSKVN
GQSGRMEFFWTILKPNDAINFESNGNFIAPEYAYKLVKKGDSTIMKSELEYGNCNTKCQTPMGAINSSMPFHNI
HPLTIGECPKYVKSNRLVLATGLRNSPQRERRGLFGAIAGFIEGGWQGMVDGWYGYHHSNEQGSGYAADKES
TQKAIDGVTNKVNSIIDKMNTQFEAVGREFNNLERRIENLNKKMEDGFLDVWTYNAELLVLMENERTLDFHD
SNVKNLYDKVRLQLRDNAKELGNGCFEFYHKCDNECMESVRSGTYDYPQYSEEARLKREEISGVKLEAAAHH
HHHHLAAEQKLISEEDLNGSKDEL

H5-ELP (Molecular weight: 101907.74 Da)
LeB4 signal peptide: **MASKPFLSLLSLSLLLFTSTCLA**
GSDQICIGYHANNSTEQVDTIMEKNVTVTHAQDILEKTHNGKLCALDGVKPLILRDCSVAGWLLGNPMCDEFI
NVPEWSYIVEKANPVNDLCYPGDFNDYEELKHLLSRINHFEKIQIIPKSSWSSHEASLGVSSACPYQGKSSFFRN
VVWLIKKNSTYPTIKRSYNNTNQEDLLVLWGIHHPNDAAEQIKLYQNPTTYISVGTSTLNQRLVPRIATRSKVN
GQSGRMEFFWTILKPNDAINFESNGNFIAPEYAYKLVKKGDSTIMKSELEYGNCNTKCQTPMGAINSSMPFHNI
HPLTIGECPKYVKSNRLVLATGLRNSPQRERRGLFGAIAGFIEGGWQGMVDGWYGYHHSNEQGSGYAADKES
TQKAIDGVTNKVNSIIDKMNTQFEAVGREFNNLERRIENLNKKMEDGFLDVWTYNAELLVLMENERTLDFHD
SNVKNLYDKVRLQLRDNAKELGNGCFEFYHKCDNECMESVRSGTYDYPQYSEEARLKREEISGVKLEGSHHH
HHHRSQLPGGQAAAEQKLISEEDLNGAVEMGHGVGVPGVGVPGGGVPGAGVPGVGVPGVGVPGVGVPGGG
VPGAGVPGGGVPGVGVPGVGVPGGGVPGAGVPGVGVPGVGVPGVGVPGGGVPGAGVPGGGVPGVGVPGVG
VPGGGVPGAGVPGVGVPGVGVPGVGVPGGGVPGAGVPGGGVPGVGVPGVGVPGGGVPGAGVPGVGVPGVG
VPGVGVPGGGVPGAGVPGGGVPGVGVPGVGVPGGGVPGAGVPGVGVPGVGVPGVGVPGGGVPGAGVPGGG
VPGVGVPGVGVPGGGVPGAGVPGVGVPGVGVPGVGVPGGGVPGAGVPGGGVPGVGVPGVGVPGGGVPGAG
VPGVGVPGVGVPGVGVPGGGVPGAGVPGGGVPGVGVPGVGVPGGGVPGAGVPGVGVPGVGVPGVGVPGGG
VPGAGVPGGGVPGVGVPGVGVPGGGVPGAGVPGVGVPGVGVPGVGVPGGGVPGAGVPGGGVPGVGVPGVG
VPGGGVPGAGVPGVGVPGVGVPGVGVPGGGVPGAGVPGGGVPGGLAAAEPKDEL

(H5pII)3 (Molecular weight: 65167.55 Da)
LeB4 signal peptide: **MASKPFLSLLSLSLLLFTSTCLA**
GSDQICIGYHANNSTEQVDTIMEKNVTVTHAQDILEKTHNGKLCALDGVKPLILRDCSVAGWLLGNPMCDEFI
NVPEWSYIVEKANPVNDLCYPGDFNDYEELKHLLSRINHFEKIQIIPKSSWSSHEASLGVSSACPYQGKSSFFRN
VVWLIKKNSTYPTIKRSYNNTNQEDLLVLWGIHHPNDAAEQIKLYQNPTTYISVGTSTLNQRLVPRIATRSKVN
GQSGRMEFFWTILKPNDAINFESNGNFIAPEYAYKLVKKGDSTIMKSELEYGNCNTKCQTPMGAINSSMPFHNI
HPLTIGECPKYVKSNRLVLATGLRNSPQRERRGLFGAIAGFIEGGWQGMVDGWYGYHHSNEQGSGYAADKES
TQKAIDGVTNKVNSIIDKMNTQFEAVGREFNNLERRIENLNKKMEDGFLDVWTYNAELLVLMENERTLDFHD

SNVKNLYDKVRLQLRDNAKELGNGCFEFYHKCDNECMESVRSGTYDYPQYSEEARLKREEISGVKLEGPKRM
KQIEDKIEEILSKIYHIENEIARIKKLIGERAAAHHHHHHLAAEQKLISEEDLNGSKDEL

(H5pII-ELP)3 (Molecular weight: 106209.84 Da)
LeB4 signal peptide: **MASKPFLSLLSLSLLLFTSTCLA**
GSDQICIGYHANNSTEQVDTIMEKNVTVTHAQDILEKTHNGKLCALDGVKPLILRDCSVAGWLLGNPMCDEFI
NVPEWSYIVEKANPVNDLCYPGDFNDYEELKHLLSRINHFEKIQIIPKSSWSSHEASLGVSSACPYQGKSSFFRN
VVWLIKKNSTYPTIKRSYNNTNQEDLLVLWGIHHPNDAAEQIKLYQNPTTYISVGTSTLNQRLVPRIATRSKVN
GQSGRMEFFWTILKPNDAINFESNGNFIAPEYAYKLVKKGDSTIMKSELEYGNCNTKCQTPMGAINSSMPFHNI
HPLTIGECPKYVKSNRLVLATGLRNSPQRERRGLFGAIAGFIEGGWQGMVDGWYGYHHSNEQGSGYAADKES
TQKAIDGVTNKVNSIIDKMNTQFEAVGREFNNLERRIENLNKKMEDGFLDVWTYNAELLVLMENERTLDFHD
SNVKNLYDKVRLQLRDNAKELGNGCFEFYHKCDNECMESVRSGTYDYPQYSEEARLKREEISGVKLEGPKRM
KQIEDKIEEILSKIYHIENEIARIKKLIGERGSHHHHHHRSQLPGGQAAAEQKLISEEDLNGAVEMGHGVGVPG
VGVPGGGVPGAGVPGVGVPGVGVPGVGVPGGGVPGAGVPGGGVPGVGVPGVGVPGGGVPGAGVPGVGVPG
VGVPGVGVPGGGVPGAGVPGGGVPGVGVPGVGVPGGGVPGAGVPGVGVPGVGVPGGGVPGAGVPG
GGVPGVGVPGVGVPGGGVPGAGVPGVGVPGVGVPGVGVPGGGVPGAGVPGGGVPGVGVPGVGVPGGGVPG
AGVPGVGVPGVGVPGVGVPGGGVPGAGVPGGGVPGVGVPGVGVPGGGVPGAGVPGVGVPGVGVPGVGVPG
GGVPGAGVPGGGVPGVGVPGVGVPGGGVPGAGVPGVGVPGVGVPGVGVPGGGVPGAGVPGGGVPGVGVPG
VGVPGGGVPGAGVPGVGVPGVGVPGVGVPGGGVPGAGVPGGGVPGVGVPGVGVPGGGVPGAGVPGVGVPG
VGVPGVGVPGGGVPGAGVPGGGVPGVGVPGVGVPGGGVPGAGVPGVGVPGVGVPGVGVPGGGVPGAGVPG
GGVPGGLAAAEPKDEL

N1 (Molecular weight: 48415.93 Da)
LeB4 signal peptide: **MASKPFLSLLSLSLLLFTSTCLA**
GSIHTGNQHQAEPISNTNFLTEKAVASVKLAGNSSLCPINGWAVYSKDNSIRIGSKGDVFVIREPFISCSHLECRT
FFLTQGALLNDKHSNGTVKDRSPHRTLMSCPVGVAPSPYNSRFESVAWSASACHDGTSWLTIGISGPDNGAVA
VLKYNGIITDTIKSWRNNILRTQESECACVNGSCFTVMTDGPSNGQASHKIFKMEKGKVVKSVELDAPNYHYE
ECSCYPDAGEITCVCRDNWHGSNRPWVSFNQNLEYQIGYICSGVFGDNPRPNDGTGSCGPVSSNGAYGVKGFS
FRYGNGVWIGRTKSTNSRSGFEMIWDPNGWTETDSSFSVKQDIVAITDWSGYSGSFVQHPELTGLDCIRPCFW
VELIRGRPKESTIWTSGSSISFCGVNSDTVGWSWPDGAELPFTIDKAAAHHHHHHLAAEQKLISEEDLNGSKDE
L

(pLI-N1)4 (Molecular weight: 52649.91 Da)
LeB4 signal peptide: **MASKPFLSLLSLSLLLFTSTCLA**
GSRMKQIEDKLEEILSKLYHIENELARIKKLLGERVDIHTGNQHQAEPISNTNFLTEKAVASVKLAGNSSLCPIN
GWAVYSKDNSIRIGSKGDVFVIREPFISCSHLECRTFFLTQGALLNDKHSNGTVKDRSPHRTLMSCPVGVAPSP
YNSRFESVAWSASACHDGTSWLTIGISGPDNGAVAVLKYNGIITDTIKSWRNNILRTQESECACVNGSCFTVMT
DGPSNGQASHKIFKMEKGKVVKSVELDAPNYHYEECSCYPDAGEITCVCRDNWHGSNRPWVSFNQNLEYQIG
YICSGVFGDNPRPNDGTGSCGPVSSNGAYGVKGFSFRYGNGVWIGRTKSTNSRSGFEMIWDPNGWTETDSSFS
VKQDIVAITDWSGYSGSFVQHPELTGLDCIRPCFWVELIRGRPKESTIWTSGSSISFCGVNSDTVGWSWPDGAE
LPFTIDKAAAHHHHHHLAAEQKLISEEDLNGSKDEL

(N1-ELP)4 (Molecular weight: 89458.22 Da)
LeB4 signal peptide: **MASKPFLSLLSLSLLLFTSTCLA**
GSIHTGNQHQAEPISNTNFLTEKAVASVKLAGNSSLCPINGWAVYSKDNSIRIGSKGDVFVIREPFISCSHLECRT
FFLTQGALLNDKHSNGTVKDRSPHRTLMSCPVGVAPSPYNSRFESVAWSASACHDGTSWLTIGISGPDNGAVA
VLKYNGIITDTIKSWRNNILRTQESECACVNGSCFTVMTDGPSNGQASHKIFKMEKGKVVKSVELDAPNYHYE
ECSCYPDAGEITCVCRDNWHGSNRPWVSFNQNLEYQIGYICSGVFGDNPRPNDGTGSCGPVSSNGAYGVKGFS
FRYGNGVWIGRTKSTNSRSGFEMIWDPNGWTETDSSFSVKQDIVAITDWSGYSGSFVQHPELTGLDCIRPCFW
VELIRGRPKESTIWTSGSSISFCGVNSDTVGWSWPDGAELPFTIDKGSHHHHHHRSQLPGGQAAAEQKLISEED
LNGAVEMGHGVGVPGVGVPGGGVPGAGVPGVGVPGVGVPGVGVPGGGVPGAGVPGGGVPGVGVPGVGVP
GGGVPGAGVPGVGVPGVGVPGVGVPGVGVPGGGVPGAGVPGGGVPGVGVPGVGVPGVGVPGGGVPGAGVPGVGVPGVGVP
GVGVPGGGVPGAGVPGGGVPGVGVPGVGVPGVGVPGGGVPGAGVPGVGVPGVGVPGVGVPGGGVPGAGVPGGGVP
GVGVPGVGVPGGGVPGAGVPGVGVPGVGVPGVGVPGGGVPGAGVPGGGVPGVGVPGVGVPGGGVPGAGVP
GVGVPGVGVPGVGVPGGGVPGAGVPGGGVPGVGVPGVGVPGGGVPGAGVPGVGVPGVGVPGGGVPGAGVP
GAGVPGGGVPGVGVPGVGVPGGGVPGAGVPGVGVPGVGVPGVGVPGGGVPGAGVPGGGVPGVGVPGVGVP
GGGVPGAGVPGVGVPGVGVPGVGVPGGGVPGAGVPGGGVPGVGVPGVGVPGGGVPGAGVPGVGVPGVGVP
GVGVPGGGVPGAGVPGGGVPGGLAAAEPKDEL

(pLI-N1-ELP)4 (Molecular weight: 93692.20 Da)
LeB4 signal peptide: **MASKPFLSLLSLSLLLFTSTCLA**
GSRMKQIEDKLEEILSKLYHIENELARIKKLLGERVDIHTGNQHQAEPISNTNFLTEKAVASVKLAGNSSLCPIN
GWAVYSKDNSIRIGSKGDVFVIREPFISCSHLECRTFFLTQGALLNDKHSNGTVKDRSPHRTLMSCPVGVAPSP
YNSRFESVAWSASACHDGTSWLTIGISGPDNGAVAVLKYNGIITDTIKSWRNNILRTQESECACVNGSCFTVMT
DGPSNGQASHKIFKMEKGKVVKSVELDAPNYHYEECSCYPDAGEITCVCRDNWHGSNRPWVSFNQNLEYQIG
YICSGVFGDNPRPNDGTGSCGPVSSNGAYGVKGFSFRYGNGVWIGRTKSTNSRSGFEMIWDPNGWTETDSSFS

VKQDIVAITDWSGYSGSFVQHPELTGLDCIRPCFWVELIRGRPKESTIWTSGSSISFCGVNSDTVGWSWPDGAE
LPFTIDKGSHHHHHHRSQLPGGQAAA**EQKLISEEDLN**GAVEMGHGVG**VPGVGVPGGGVPGAGVPGVGVPGV**
GVPGVGVPGGGVPGAGVPGGGVPGVGVPGVGVPGGGVPGAGVPGVGVPGVGVPGVGVPGGGVPGAGVPGG
GVPGVGVPGVGVPGGGVPGAGVPGVGVPGVGVPGVGVPGGGVPGAGVPGGGVPGVGVPGVGVPGGGVPGA
GVPGVGVPGVGVPGVGVPGGGVPGAGVPGGGVPGVGVPGVGVPGGGVPGAGVPGVGVPGVGVPGVGVPGG
GVPGAGVPGGGVPGVGVPGVGVPGGGVPGAGVPGVGVPGVGVPGVGVPGGGVPGAGVPGGGVPGVGVPGV
GVPGGGVPGAGVPGVGVPGVGVPGVGVPGGGVPGAGVPGGGVPGVGVPGVGVPGGGVPGAGVPGVGVPGV
GVPGVGVPGGGVPGAGVPGGGVPGVGVPGVGVPGGGVPGAGVPGVGVPGVGVPGVGVPGGGVPGAGVPGG
GVPGVGVPGVGVPGGGVPGAGVPGVGVPGVGVPGVGVPGGGVPGAGVPGGGVPGGLAAAEP**KDEL**

100xELP (Molecular weight: 44280.75 Da)
LeB4 signal peptide: **MASKPFLSLLLSLSLLLFTSTCLA**
GSHHHHHHRSQLPGGQAAA**EQKLISEEDLN**GAVEMGHGVG**VPGVGVPGGGVPGAGVPGVGVPGVGVPGVG**
VPGGGVPGAGVPGGGVPGVGVPGVGVPGGGVPGAGVPGVGVPGVGVPGVGVPGGGVPGAGVPGGGVPGVG
VPGVGVPGGGVPGAGVPGVGVPGVGVPGVGVPGGGVPGAGVPGGGVPGVGVPGVGVPGGGVPGAGVPGVG
VPGVGVPGVGVPGGGVPGAGVPGGGVPGVGVPGVGVPGGGVPGAGVPGVGVPGVGVPGVGVPGGGVPGAG
VPGGGVPGVGVPGVGVPGGGVPGAGVPGVGVPGVGVPGVGVPGGGVPGAGVPGGGVPGVGVPGVGVPGGG
VPGAGVPGVGVPGVGVPGVGVPGGGVPGAGVPGGGVPGVGVPGVGVPGGGVPGAGVPGVGVPGVGVPGVG
VPGGGVPGAGVPGGGVPGVGVPGVGVPGGGVPGAGVPGVGVPGVGVPGVGVPGGGVPGAGVPGGGVPGVG
VPGVGVPGGGVPGAGVPGVGVPGVGVPGVGVPGGGVPGAGVPGGGVPGGLAAAEP**KDEL**

VII.2 Comparison of consensus amino acid sequences of three hemagglutinins.

Table VII-1. Percent similarity in amino acid sequence.

	A/Hatay/2004(H5N1) with HA accession Q5QQ29
A/swan/Germany/R65/2006(H5N1) with HA accession ABE26829	96
A/duck/Vietnam/TG24-01/05(H5N1) with HA accession Q14RX0	98.6

Three amino acid sequences of influenza A hemagglutinins belonging to three different strains (A/Hatay/2004(H5N1), A/swan/Germany/R65/2006(H5N1) and A/duck/Vietnam/TG24 -01/05(H5N1)) were compared by clustal W method. As can be seen from table VII-1, hemagglutinins from the challenge H5 avian influenza virus (A/duck/Vietnam/TG24-01/05 (H5N1)) and the inactivated H5 avian influenza virus (A/swan/Germany/R65/2006(H5N1)) had 98.6% and 96% deduced hemagglutinin amino acid sequence similarity with the recombinant H5 vaccine of (A/Hatay/2004(H5N1)) strain (Table VII-1), respectively. The HP H5N1 influenza viruses isolated in 2004, 2005 from humans and birds in Thailand, Vietnam and Cambodia were grouped to clade 1 (Adams and Sandrock, 2010; WHO/OIE/FAO H5N1 Evolution Working; Who, 2005; Yen and Webster, 2009), while A/swan/Germany/R65/2006(H5N1) strain belongs to clade 2.2.2 (Starick *et al*., 2008).

VII.3 Purification of the 100xELP tag by affinity chromatography

In order to assess an immunomodulatory role of the ELP by ELISA, the 100xELP protein (amino acid sequence was shown in section VII.1) was expressed transiently in *N. benthamiana* and purified by affinity chromatography. The Coomassie blue stained SDS-PAGE gel shows that the 100xELP protein was successfully purified by IMAC (Fig. VII-1). This protein was used for ELISA experiments in section III.8.4.

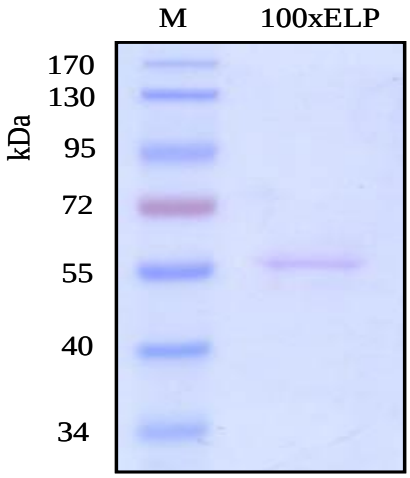

Figure VII-1. Purification of the 100xELP tag by IMAC. Purified 100xELP protein (1 µg) was separated by 10% SDS-PAGE. Proteins were detected by Coomassie staining. M: protein marker, 100xELP: the 100xELP tag protein.

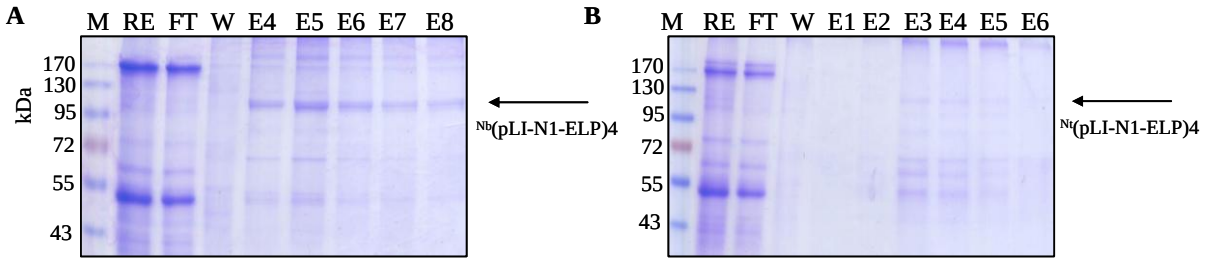

Figure VII- 2. Purification of the tetrameric (pLI-N1-ELP)4 by IMAC. 20 µl of raw extract (RE), flow through (FT) and wash fraction (W) and 40 µl of elution fractions (E4 to E8) were separated by 10% SDS-PAGE. (A) ^{Nb}(pLI-N1-ELP)4 detection by Coomassie blu stain. (B) ^{Nt}(pLI-N1-ELP)4 detection by Coomassie stain. 20 µl of raw extract (RE), flow through (FT) and wash fraction (W) and 50 µl of elution fractions (E1 to E6) were separated by 10% SDS-PAGE. ^{Nb}(pLI-N1-ELP)4 and ^{Nt}(pLI-N1-ELP)4: the tetrameric (pLI-N1-ELP)4 was purified from the transiently transformed *N. benthamiana* and stably transformed *N. tabacum* leaves, respectively.

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List of publications

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Phan, H.T., and Conrad, U. (2011) Membrane-based inverse transition cycling: An improved means for purifying plant-derived recombinant protein-elastin-like polypeptide fusions. *Int. J. Mol. Sci.* **12**(5)**:** 2808-2821.

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Phan, H.T. Influenza virus-like particles (VLPs) comprising hemagglutinin produced within a plant. Presentation in the frame of PhD seminar, 9th September, 2010, IPK, Gatersleben.

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DECLARATION

Hereby, I declare that all the work presented in this dissertation is my own, carried out solely with the help of the literature and the aids cited.

Gatersleben, April 2012 Phan Trong Hoang