UNIVERSIDAD AUTONOMA DE AGUASCALIENTES

CENTRO DE CIENCIAS BÁSICAS

DEPARTAMENTO DE QUÍMICA

"PRODUCTION OF A POULTRY VACCINE FOR NEWCASTLE DISEASE USING TRANSIENT EXPRESSION IN TOBACCO"

PRESENTADO POR:

Biól. Oscar Antonio Ortega Rivera

GRADO A OBTENER: MAESTRÍA EN CIENCIAS EN EL ÁREA DE BIOTECNOLOGÍA VEGETAL

TUTORES:

PhD. Karen McDonald

DR. José Francisco Morales Domínguez

MIEMBRO DEL COMITE TUTORAL:

DR. Eugenio Martín Pérez Molphe Balch

Aguascalientes, Ags. 23 de Julio del 2012

UNIVERSIDAD AUTONOMA DE AGUASCALIENTES

Centro de Ciencias Básicas

SR. OSCAR ANTONIO ORTEGA RIVERA,
ALUMNO (A) DE LA MAESTRÍA EN CIENCIAS EN EL ÁREA DE BIOTECNOLOGÍA VEGETAL.
P R E S E N T E .

Estimado (a) alumno (a) Ortega:

Por medio de este conducto me permito comunicar a Usted que habiendo recibido los votos aprobatorios de los revisores de su trabajo de tesis y/o caso práctico titulado: **"Production of a Poultry Vaccine for Newcastle Disease Using Transient Expression in Tobacco"**, hago de su conocimiento que puede imprimir dicho documento y continuar con los trámites para la presentación de su examen de grado.

Sin otro particular me permito saludarle muy afectuosamente.

A T E N T A M E N T E
Aguascalientes, Ags., 4 de julio de 2012
"SE LUMEN PROFERRE"
LA DECANO

M. en C. MARTHA CRISTINA GONZÁLEZ DÍAZ

UNIVERSIDAD AUTONOMA DE AGUASCALIENTES
CENTRO DE CIENCIAS BÁSICAS

c.c.p.- Archivo
MCGD,mjda

UNIVERSITY OF CALIFORNIA, DAVIS

BERKELEY · DAVIS · IRVINE · LOS ANGELES · MERCED · RIVERSIDE · SAN DIEGO · SAN FRANCISCO · SANTA BARBARA · SANTA CRUZ

DEPARTMENT OF CHEMICAL ENGINEERING
AND MATERIALS SCIENCE
TELEPHONE (530) 752-0400
FAX (530) 752-4033

ONE SHIELDS AVENUE
DAVIS, CALIFORNIA 95616-5294

M. en C. Martha Cristina González Díaz
Decana del Centro de Ciencias Básicas
PRESENTE:

July/2012

Estimada M. en C, González Díaz:

Por este medio le comunico que el **Biól. Oscar Antonio Ortega Rivera,** egresado de la Maestría en Ciencias en el Área de Biotecnología Vegetal, ha concluido satisfactoriamente su trabajo de tesis titulado: **"PRODUCTION OF A POULTRY VACCINE FOR NEWCASTLE DISEASE USING TRANSIENT EXPRESSION IN TOBACCO".**

Asimismo, le informo que después de revisar el escrito derivado del trabajo mencionado, considero que ya tiene una versión final satisfactoria y se puede proceder al examen de grado.

Respectfully,

Karen McDonald

Karen McDonald
Advisor
Professor, Chemical Engineering and Materials Science
Associate Dean for Graduate Studies and Research, College of Engineering

UNIVERSIDAD AUTONOMA DE AGUASCALIENTES

M en C. Martha Cristina González Díaz
Decana del Centro de Ciencias Básicas
P r e s e n t e:

Por medio de este conducto, le comunico el alumno **Oscar Antonio Ortega Rivera,** egresado de la Maestría en Ciencias en el área de Biotecnología Vegetal, ha concluido satisfactoriamente su trabajo de tesis titulado **"PRODUCTION OF A POULTRY VACCINE FOR NEWCASTLE DISEASE USING TRANSIENT EXPRESSION IN TOBACCO",** que defenderá en el examen de titulación correspondiente.

Por lo anterior se considera que puede proceder a realizar los trámites administrativos correspondientes y se le asigne la fecha de examen de grado.

ATENTAMENTE
"SE LUMEN PROFERRE"
Aguascalientes, Ags. A 3 de julio de 2012

Dr. Francisco Morales Domínguez
Tutor responsable

UNIVERSIDAD AUTONOMA
DE AGUASCALIENTES

M. en C. Martha Cristina González Díaz
Decana del Centro de Ciencias Básicas
P R E S E N T E :

Estimada M. en C. González Díaz:

Por este medio le comunico que el **Biol. Oscar Antonio Ortega Rivera**, egresado de la Maestría en Ciencias en el Área de Biotecnología Vegetal, ha concluido satisfactoriamente su trabajo de tesis titulado: **"PRODUCTION OF A POULTRY VACCINE FOR NEWCASTLE DISEASE USING TRANSIENT EXPRESSION IN TOBACCO"**.

Asimismo, le informo que después de revisar el escrito derivado del trabajo mencionado, considero que ya se tiene una versión final satisfactoria y se puede proceder al examen de grado.

A T E N T A M E N T E
"SE LUMEN PROFERRE"
Aguascalientes, Ags. a 03 de Julio de 2012

Dr. Eugenio Pérez Molphe Balch
Miembro del Comité Tutoral
Departamento de Química
Centro de Ciencias Básicas

AGRADECIMIENTOS

Dedicado a mis padres, hermanos, esposa, hijos y amigos
por brindarme su amor y apoyo para realizar mis sueños.

Este trabajo fue realizado solamente gracias al apoyo y guía de muchas personas. Primeramente, me gustaría agradecer a la Dra. Karen McDonald por haberme aceptado en su grupo de investigación, asi como por darme su confianza, experiencia y consejo en los momentos difíciles. Ademas por financiar mí asistencia al congreso New Cell New Vaccines VI y al evento de emprededores organizado por la NCIIA.

Tambien quiero agradecer a los miembros de mi comité tutoral, Dr. Francisco Morales y Dr. Eugenio Perez-Molphe, a quienes agradezco su apoyo y comprensión durante la realización de este proyecto. Especialmente al Dr. Francisco quien me ha brindado su amistad, y a quien debo la realización de mi estancia en la Universidad de California, Davis.

Estoy muy agradecido con el Dr. Dandekar (UC Davis) por haberme permitido realizar una gran parte de mis experimentos en su laboratorio, durante mi estancia en UC Davis. Y especialmente quiero agradecer a Sandra Uratsu (Sandie) por todo su apoyo, consejo, paciencia y sobre todo por su amistad durante el tiempo que estuve en el laboratorio del Dr. Dandekar. Sin su ayuda probablemente este trabajo no se hubiera terminado en teimpo y forma. Ademas, agradecer a la Dra. Ana Maria Ibañez, por facilitar mi corta estancia por ese laboratorio y hacerme sentir como en casa. Tambien agradezco por el apoyo técnico brindado por My Phu.

Agradezco al Dr. Falk y a la Dra. Hwang por facilitarme el plasmido y los primers correspondientes al sistema de expresión CMVva, sin los cuales mi trabajo no se habría realizado.

Quiero agradecer a mis compañeros de laboratorio en el laboratorio de la Dra. Karen, con quienes compartí muy buenos momentos. Gracias Sang-Kyu Jung, Nathaniel Kingsbury (Nate), Ingrid Leth, Salem Mohammed Al-Kanaimsh, Mark Lemos, Kittipong Rattanaporn (Nat) nunca olvidare el tiempo que compartimos en el laboratorio y fuera de el. Especialmente quiero agradecer a Lucas Arzola, quien desde que nos conocimos me brindo su amistad durante toda mi estancia en UC Davis, además de siempre estar allí para apoyarme física o moralmente en mis experimentos, por darme la oportunidad de integrarme a su equipo emprendedor “Inserogen”. Tambien a mi gran amiga desde la licenciatura Alexandra Ramirez (Lokita), por su amistad, consejo y alegría en los momentos difíciles, y por ser quien me alentó a realizar la estancia en UC Davis. Y bueno a todos las personas maravillosas que conoci en Davis y a quienes agradezco hacer de mi estancia una experiencia inolvidable, gracias: Denisse, Carlos

(Porro), Stephany, Michelle, Pedro, Coni, Santi, Rogelio, Gerardo (Gerry), Cush, Javiercito, Felipe, Eliana, Gerardo (Chilango), Adriana, Janette, y a todos los colados en las fiestas en Davis.

Tambien quiero agradecer a los miembros del departamento de Ingenieria Quimica de UC Davis, Jim Gage (Engineering Lab Manager), Carole Bustamante (Contracts & Grants Manager), Veronica Stanton (Financial Assistant). Todos ayudaron a que mi estancia fuera muy agradable y sin problemas.

Agradezco enormemente a mis padres y hermanos, por siempre apoyarme en todas las etapas de mi vida y brindarme su amor y consejo. A mi esposa Marisela e hijos (Álister y André), con los que siempre estare agradecido por haber soportado mi ausencia por seis meses y seguir a mi lado. Especialmente a mi esposa, por cuidar y amar a nuestros hijos como solo ella podría hacerlo. Por tolerarme durante todos estos años, por todo su amor y comprensión y por siempre apoyarme en mis locuras. Por todo eso y más te amo Marisela (mi Pke).

Finalmente agradezco a CONACYT por brindarme la beca para realizar mis estudios de posgrado, asi como una beca-mixta para la realización de mi estancia en UC Davis. Además agradezco a la Decano M. en C. Martha Cristina Gonzalez y al Depto de Intercambio y Becas de la UAA, por brindarme el apoyo económico que me hacia falta para hacer mi estancia en UC Davis.

ABSTRACT

Newcastle disease (ND) is caused by the ND virus (NDV) or avian paramyxovirus serotype-1 (AMPV-1) and is considered as the most important disease among the poultry industry worldwide (Alexander 2003). In Mexico, it is a disease with greater economic and health significance in the Mexican poultry industry, since the early 50's. NDV belong to the Paramyxoviridae family and as the most of Paramyxoviridae's members the ND virion contains two types of surface glycoproteins, the fusion (F) protein and the hemagglutinin-neuraminidase (HN) protein, which are the major antigenic determinants and epitopes that stimulate the production of virus-neutralizing antibodies have been determined for both F (Toyoda et al 1988) and HN (Chambers et al 1988; Iorio et al 1991). The practice of vaccination for the prevention of animal disease has been used for centuries and has proven to be a powerful tool for the alleviation of animal suffering as well as the economic well being of producers of animal products. Although vaccine technology has made substantial progress, the basic concept remains the same. Most of the currently vaccines used are composed of either inactivated or live lentogenic strains of NDV. However, live vaccines are infectious and the dead vaccines may induce fears of disease emergence (Gallili and Ben-Nathan 1998; OIE 2010). This problem can be overcome with the use of subunit vaccines, which are composed only with antigenic protein or epitope of the virus or bacteria. Recently, plants have been investigated as a source for the production of therapeutic agents such as vaccines, antibodies, and biopharmaceuticals. These plant expression systems provide several advantages such as lack of risk of contamination with animal pathogens, a heat-stable environment, and would avoid injection-related hazards if administered as an edible agent (Thanavala et al 2006). In addition, there has been an increasing trend towards the use of transient expression systems in recent years, mostly in plant-made vaccines. The major reason for this is sheer convenience and speed. In this study we attempted to transient express the HN protein in harvested *Nicotiana benthamiana* leaves using vacuum agroinfiltration to deliver the exogenous gene. The cDNA of a NDV strain isolated from the last outbreak in California, US (CA02) was engineered and codon optimized to get four different constructs (HNop1, 2, 3 and 4), which were synthesized *de novo* and cloned in three different expression systems (TRBO, CMVva and 35S). The kinetic expression was measured at 2, 4 and 6 day post-infiltration (DPI) by ELISA. After the screening of expression systems, HNop constrcusts, and DPIs significance differences ($p<0.05$) were found. Where, the higher

yield was obtained at DPI 6 using the TRBO expression system carryng the HNop1 construct (~20mg/kg FW). The expected molecular weight of the plant-made HN protein (~60 kDa) was confirmed through Western blot assay in all the samples. Most important, in this study we demonstrated that using the platform SwiftVax® in six days we can produce up to 1000-1300 doses of 15-20µg. Therefore, future outbreaks can be overcome producing subunit vaccines in days instead of months.

RESUMEN

La enfermedad de Newcastle (ND) es causada por el virus NDV o paramyxovirus aviar serotipo-1 (AMPV-1) y es considerada como la mas importante enfermedad en la industria avícola alrededor del mundo (Alexander 2003). En México, es una enfermedad con gran significancia económica y de salud para la industria mexicana avícola, desde principios de los 50's. El NDV pertenece a la familia Paramyxoviridae y como la mayoría de los miembros de esta familia el virion de ND contiene dos tipos de glicoproteínas de superficie, la proteina de fusión (F) y la proteina hemaglutinina-neuraminidasa (HN), las cuales son los mayores determinantes antigénicos y los epítopos que estimulan la producción de anticuerpos neutralizantes de virus ya han sido determinados para ambas proteínas F (Toyoda et al 1988) y HN (Chambers et al 1988; Iorio et al 1991). La practica de la vacucnacion para la prevención de enfermedades animales ha sido usada por cientos de años y ha probado ser un arma poderosa para disminuir el sufrimiento animal, asi como también el bien económico de productores de productos animales. A pesar de que la tecnología de las vacunas ha hecho progresos sustanciales, el concepto básico sigue siendo el mismo. La mayoría de las vacunas actuales están compuestas de cepas de NDV, ya sea inactivadas o lentogénicas (poco virulentas) vivas. Sin embargo, las vacunas vivas son infecciosas y las inactivadas pueden inducir miedo de una posible aparición de la enfermedad (Gallili and Ben-Nathan 1998; OIE 2010). Este problema puede ser resuelto con el uso de vacunas de subunidad, las cuales están compuestas solo con la proteina antigénica o el epitopo del virus o bacteria. Recientemente, las plantas han sido investigadas como una fuente para la producción de agentes terapéuticos tales como vacunas, anticuerpos y biofarmacéuticos. Estos sistemas de expresión con plantas proveen muchas ventajas tales como la falta de riesgo de contaminación con patógenos animales, ambientes con calor estable y puede evitar peligros relacionados a la inyección si son admisnistrados como agentes comestible (Thanavala et al 2006). Adicionalmente, ha habido una tendencia creciente hacia el uso de sistemas de expresión transitoria en años recientes, principalmente para vacunas producidas en plantas. La mayor razón de esto es meramente por conveniencia y velocidad. En este estudio, nosotros intentamos expresar transitoriamente la proteina HN en hojas cosechadas de *Nicotiana benthamiana*, usando agroinfiltración con vacio para la entrega del gen exógeno. El ADNc de una cepa del NDV aislada del ultimo brote en California, US (CA02) fue modificada y los codones se optimizaron para obtener cuatro

diferentes construcciones (HNop1, 2, 3 y 4), las cuales fueron sintetizadas *de novo* y clonadas en tres diferentes sistemas de expresión (TRBO, CMVva y 35S). La cinetica de expresión fue medida los días 2, 4 y 6 post infiltración (DPI) mediante ELISA. Después del escrutinio de los sistemas de expresión, las construcciones HNop y los DPIs diferencias significativas ($P<0.05$) fueron encontradas. Donde, el rendimiento mas alto fue obenido en el DPI 6 usando el sistema de expresión TRBO con la construcción HNop1 (~20mg/kg PF). El peso molecular esperado de la proteina HN expresada en planta (~60 kDa) fue confirmado a través de un ensayo de Western blot en todas las muestras. Lo mas importante, en este estudio demostramos que usando la plataforma de biofabricacion SwiftVax® en seis días podemos producir hasta 1000-1300 dosis de 15-20µg. Por lo tanto, futuros brotes pueden ser sobre llevados produciendo vacunas de subunidad en días en lugar de meses.

GENERAL INDEX

Page

GENERAL INDEX 1
TABLES INDEX 4
FIGURES INDEX 5
INTRODUCTION 7
1. BACKGROUND 8
1.1 NEWCASTLE DISEASE 8
1.1.1 History and distribution 8
1.1.2 Clinical and pathology features 11
1.1.3 Taxonomy and classification 12
1.1.4 Molecular features 16
1.1.5 Viral Entry into Cells 19
1.1.6 Pathogenesis 20
1.1.7 NDV attachment proteins 21
1.1.8 Transmission 24
1.1.9 Public health 25
1.1.10 Prevention of Newcastle disease 25
1.1.11 Vaccination side effects 29
1.1.12 Importance of NDV strains in vaccine formulation 30
1.2 PRODUCTION OF ANTIGENS IN PLANTS 31
1.2.1 Plant-made vaccines 31
1.2.2 Genetic transformation methods 36
1.2.3 Transient expression and agroinfiltration 39
1.2.4 Anriviral RNA silencing and viral silencing RNA suppressors 44
1.2.5 Codon optimization 47
2. JUSTIFICATION 49
3. HYPOTHESIS 50
4. GENERAL OBJECTIVE 50

4.1 PARTICULAR OBJECTIVES 50

5. METHODOLOGY 51

5.1 IN SILICO DESIGN OF THE HN GENE CONSTRUCTS FOR TRANSIENT EXPRESSION IN NICOTIANA BENTHAMIANA 51

5.1.1 Selection of NDV strain to express the HN protein 51

5.1.2 Codon optimization of HN gene sequence and additional elements 52

5.2 GROWTH OF NICOTIANA BENTHAMIANA PLANTS 53

5.2.1 Preparation of Nicotiana benthamiana plants 53

5.3 CLONING OF HNop CONSTRUCTS INTO THE p35S, pCMVva AND pTRBO BINARY VECTORS, VERIFICATION OF CORRECT INSERTION AND TRANSFER INTO *AGROBACTERIUM TUMEFACIENS* 53

5.3.1 Transformation of competent cells with pUC57:HNop1-4 54

5.3.2 Cloning of HNop1-4 constructs into p35S binary vector 54

5.3.3 Cloning of HNop1-4 constructs into pTRBO binary vector 59

5.3.4 Cloning of HNop1-4 constructs into pCMVva binary vector 62

5.4 VACUUM AGROINFILTRATION OF HARVESTED *NICOTIANA BENTHAMIANA* LEAVES 64

5.4.1 Growth of recombinant *Agrobacterium*, agroinfiltration and incubation conditions 64

5.5 PROTEIN EXPRESSION KINETIC OF AGROINFILTRATED *NICOTIANA BENTHAMIANA* LEAVES 67

5.5.1 Sampling and HN protein extraction 67

5.5.2 Bradford analysis of total soluble protein (TSP) 67

5.6 HN PROTEIN EXPRESSION LEVEL IN AGROINFILTRATED *NICOTIANA BENTHAMIANA* LEAVES 68

5.6.1 ELISA analysis 68

5.7 CHARACTERIZATION OF HN PROTEIN 68

5.7.1 Western blot assay 68

5.8 STATISTICAL ANALYSIS 69

6. RESULTS 69

6.1 SELECTION OF NDV STRAIN AND ENGINEERING OF HN cDNA TO BE TRANSIENTLY EXPRESSED 69

6.1.1 Multiple sequence alignment for HN protein of different NDV strains..... 69

6.1.2 Engineering and codon optimization of HN cDNA 70

6.1.3 Cloning of HNop constructs into p35S, pTRBO and pCMVva binary vector 71 72

6.2 TRANSIENT EXPRESSION OF HN PROTEIN IN *NICOTIANA BENTHAMIANA* LEAVES 74

6.2.1 Growth of Nicotiana benthamiana plants 74

6.2.2 Vacuum-agroinfiltration of harvested leaves..... 75

6.2.3 Sampling of agroinfiltrated leaves 75

6.2.4 Bradford assay for total soluble protein (TSP)..... 77

6.2.5 ELISA assay and percentage of total soluble HN protein..... 79

6.2.6 Western blot assay..... 82

7. DISCUSSION 83

8. CONCLUSIONS..... 90

9. FUTURE DIRECTIONS 91

10. GLOSSARY 91

11. REFERENCE 97

ANNEX 112

ANNEX A

ANNEX B

ANNEX C

TABLES INDEX

Page

Table 1.1 Comparison of recombinant protein production in different systems 33

Table 1.2 Subunit vaccine candidates for animal infectious disease control expressed by transgenic plants, plant cell cultures or plant viruses 34

Table 1.3 Transient expression of vaccine antigens in plants 40

Table 1.4 Plant viral suppressors of RNA silencing 45

Table 5.1 Sequence of the primers used to perform the colony PCR 59

Table 5.2 Growth conditions for all binary vectors generated and transformed into *Agrobacterium tumefaciens* 65

Table 6.1 Codon optimization of HN constructs 71

FIGURES INDEX

Page

Figure 1.1 Current status of occurrence of velogenic Newcastle disease (vND) in Mexico 10

Figure 1.2 Clinical and pathologic features of Newcastle disease virus (NDV)..... 12

Figure 1.3 Phylogenetic tree of representative members of the *Paramyxoviridae* family..... 15

Figure 1.4 Phylogenetic tree for avian paramyxovirus-1..... 16

Figure 1.5 Molecular composition of paramyxoviruses 18

Figure 1.6 Schematic of different pathways of viral entry..... 20

Figure 1.7 Structure of the NDV HN ectodomaim 23

Figure 1.8 Summary of major molecular events and structures within the *Agrobacterium* cell..... 39

Figure 1.9 Current model of antiviral RNA silencing in plants and its suppression by virus-encoded silencing suppressors 46

Figure 5.1 Plasmid map of the commercial vector pUC57-Amp 54 without the HNop constructs..... 53

Figure 5.2 Plasmid DNA of pUC57:HNop1-4 and pDE00.0113 digested with XhoI and HindIII restriction enzymes..... 55

Figure 5.3 Plasmid maps used to make the 35S binary vector 58

Figure 5.4 Plasmid map of pJL TRBO binary vector..... 60

Figure 5.5 Plasmid DNA of pUC57:HNop1-4 and pJL TRBO digested with PacI and AvrII restriction enzymes 61

Figure 5.6 Plasmid map of Cucumber Mosaic Virus viral amplicon (CMVva) system..... 63

Figure 5.7 Plasmid DNA of pUC57:HNop1-4 and pMCSdelQAN digested with PstI and HindIII restriction enzymes..... 63

Figure 5.8 Scheme of the biomanufacturing platform SwiftVax®..... 66

Figure 6.1 Multiple sequence alignment of NDV-HN protein sequence of different strains..... 70

Figure 6.2 Schematic diagram of optimized HN (HNop) constructs synthesized..... 71

Figure 6.3 Testing of binary vectors with the HNop constructs 72

Figure 6.4 Maps of the T-DNA region of engineered binary vectors 74

Figure 6.5 *N. benthamiana* plants grown in greenhouse conditions..... 75

Figure 6.6 Harevested N. benthamiana leaves..... 75

Figure 6.7 Sampling of agroinfiltrated leaves 77

Figure 6.8 Measuring of total soluble protein (TSP) expression..... 79

Figure 6.9 Concentration of plant-expressed HN protein in agroinfiltrated leaves 81

Figure 6.10 Percentage of HN protein in total soluble protein (TSP) in agroinfiltrated leaves 82

Figure 6.11 Western blot analysis of agroinfiltrated *Nicotiana benthamiana* leaves..... 83

INTRODUCTION

Chickens have been bres in captivity in Egypt since about 1400 BC. The red jungle fowl, an Asian breed, is assumed to be the ancestor of our modern poultry breeds.The warm regions of the world were the areas from which all modern breeds of chicken have evolved. Poultry were kept by farmers in China, India and East Asia long before they were known to the Europeans and Americans. Poultry as a business, however, was not known before the twentieth century. It has in recent years occupied a leading role among agricultural industries in many parts of the world. There is a remarkable growth in poultry meat (436.5%) and eggs (203.2%) during the last 35 years. These products have increased much fater than beef and veal (57.6%) or pig meat (186.4%) (Daghir 2008). The potential for further growth is obvious in view of the value of eggs and poultry meat as basic protective foods in the human diet. Africa, Asia and South America show the greatest increases in egg production, with decreases in both Europe and Oceania. Chicken meat production continued to increase in all continents, with the highest increase in Asia and South America. The worldwide rate of increase in egg production has averaged 5.3% per year, while for chicken meat it was slightly higher at 5.7% per year (Daghir 2008). Additionaly, poultry meat and eggs are among the highest-quality humand foods; they can serve as important sources of animal protein in those areas of the world that have protein insufficiency. Most countries in the hot regions of the world have daily per capita animal protein consumption below that recommended by the Food and Agriculture Organization (FAO) and the World Health Organization (WHO). Commercial poultry development has been occurring in a number of Latin American countries, particularly Brazil, Chile, Colombia, Mexico and Venezuela. Per capita egg consumption in Mexico today is 22.8 kg and 26.13 kg of chicken meat (UNA 2012). Moreover, the egg and chicken meat production increased since 1994 to date 69% and 104%, respectively (UNA 2012). Therefore, there is no doubt that this increase in the availability of eggs and poultry meat will contribute significantly to the improvement of the nutritional status of the people in the developing countries. Although the need for more eggs and poultry meat is obvious and the availability of these products can go a long way to meet the protein needs of several populations in hot regions, there are several constraints to the future development of the poultry industry. One of them constraints is the control of avian diseases that can decline the poultry production. Among the most important poultry diseases are Avian Influenza and Newcastle Disease, which are responsible of the major of economic losses in the poultry industry worldwide. For example, in 2002-03 poultry industry in

California suffered the effect of a Newcastle disease outbreak, where more than 3 million birds where depopulated and the economic losses reached up to $360 million. More recently, in July at Jalisco state, Mexico, was reported an avian influenza outbreak in layer hens, which 2.5 millions has been euthanized and economic losses are around $50 million (SAGARPA 2012). Therefore, mainly prevention strategies are required for well functioning of poultry industry, such as vaccination that has been used for centuries and has proven to be a powerful tool for the alleviation of animal suffering as well as the economic well being of producers of animal products. Keeping this in mind, the development of a new biomanufacturing plant-based platform, to the production of a poultry vaccine against Newcastle disease, was accomplished in the present study.

1. BACKGROUND

1.1 NEWCASTLE DISEASE

1.1.1 History and distribution

Newcastle disease (ND) is a viral disease of birds with a wide range of clinical signs ranging from mild to severe and is caused by avian paramyxovirus serotype-1 (AMPV-1). The term “Newcastle disease” was coined by Doyle as a temporary name to distinguish it from other diseases at the time (Alexander et al 2004; Saif et al 2008). The name was never changed, but APMV-1 has become an alternative term used interchangeably with ND virus (NDV) (Kim et al 2008). Less virulent strains are endemic to the United States while highly virulent strains are endemic to Asia, Middle East, Africa, Central and South America and parts of Mexico. The highly virulent form of the disease is one of the most important in poultry worldwide. Chickens are particularly susceptible and may experience morbidity and mortality rates of up to 100%. The most virulent outbreaks of ND have an enormous impact on backyard poultry in developing countries, where these birds are an important source of protein. Although the disease caused by low pathogenic strains can decrease productivity, since it is common in poultry worldwide it does not have a significant impact on international trade (Alexander 1991; Beard and Hanson 1988). In developed countries, where the most virulent forms of the virus have been eradicated, trade embargoes and restrictions cause major economic losses, during an outbreak (Alexander 1991; Beard and Hanson 1988). Due the United States (US) is the most important producer of chicken meat with around 15 millions of tons per year (FAO 2010); current outbreaks have been a serious impact in this productivity sector. The 2002-2003 outbreak in

California caused the most significant economic loss resulting in the depopulation of more than 3 million birds on 2,671 premises including 21 commercial table-egg layer flocks (Saif et al 2008; Perozo et al 2008b). Transportation of infected birds or contaminated material and transmissibility of the disease led to subsequent outbreaks in Nevada, Arizona, and Texas. Efforts to eradicate the disease cost the US an estimated $180 to $360 million (Kim et al 2008).

The virulent form of NDV was first discovered in Java, Indonesia, and Newcastle upon Tyne region in England in 1926 (Alexander et al 2004; Saif et al 2008; Seal et al 2000; Li et al 2009). Historical data indicate that outbreaks in poultry with symptoms similar to those seen with virulent ND may have been present in Korea prior to 1926 and also in Scotland as early as 1896. According to Hanson (Cited by Hines and Miller 2012), there are three hypotheses to explain the sudden occurrence of virulent ND in Southeast Asia. First, it is possible that virulent ND was endemic in Southeast Asia and only became a problem when poultry became commercialized (Alexander et al 2004). The second theory is that virulent ND was present in bird species living in the tropical rain forest and was introduced into poultry by man similar to the way the movement of tropical birds spread the disease today. The third explanation is that a major mutation occurred in the precursor virus allowing for a change in pathogenicity from low virulence to high virulence. Having the ability to infect all orders of avian species, APMV-1 has been able to spread throughout the world resulting in four panzootics (Alexander et al 2004; Kim et al 2008; Seal et al 2005; Wakamatsu et al 2006; Berinstein et al 2008). The initial panzootic took 20 years to develop spreading very slowly throughout the world (Alexander et al 2004). The US was likely not involved in the first panzootic but was not so lucky during the second panzootic. The second outbreak spread at a much faster rate, taking only 4 years to spread throughout the world. Globalization and the development of various modes of transportation led to the increased rate of disease spread during the second, third, and fourth panzootics occurring in 1960, the late 1970s, and the 1980s, respectively (Hines and Miller 2012).

In Mexico, it is a disease with greater economic and health significance in the Mexican poultry industry, since the early 50's. Probably the disease agent was introduced to the country prior to 1950, however, such as the poultry industry at that time consisted of backyard poultry, from 50 to 1000 birds, when the disease was present in some of them, the losses caused on account of high mortality and a decrease in the production, never considered as losses of great magnitude, a situation that changed after 1952-

1953, when the poultry industry began to perform as an industry with high production of egg and roasting chicken (fourth place in chicken meat production, close to 3 millions of tons per year [FAO 2010]), with poultry farms from 50 to 200 thousand birds; it then became essential to protect each farm against ND (Botero 2006). The current regulations for the control of the disease lies in the NOM-013-ZOO-1994, published in the Official Journal of the Federation on February 28, 1995. The purpose of this rule is to eliminate the velogenic or virulent ND in the country, mainly through preventive strategies. In 2012 the Ministry of Agriculture, Livestock, Rural Development, Fisheries and Food (SAGARPA) has reported that the disease is almost controlled in all the country, with only one states under process of eradication (Figure 1.1). Locally, the state of Aguascalientes is free of NDV thanks to prevention program achieved during all the production process. It occupies the fourth place in production (10-12%) in Mexico, with an annual economic growth of 3.5-4% and is considered the region with the most production per squared kilometer worldwide (UNA 2012).

Although the most significant impact of the disease is in chickens, it is known that ND viruses infect more than 250 species of birds in 27 orders; other avian species may also be susceptible. It can also cause infections in humans, but has not been reported in other mammalian species. Some domestic and zoo birds get sick after infection, while other species can be carriers and shed virulent virus without symptoms. These birds, especially psittacines imported illegally, can introduce ND in disease-free countries. Most of the viruses found in wild birds are lentogenic (asymptomatic), but the virulent have been established in some populations of cormorants (*Phalacrocorax spp*; Order Pelecaniformes) and cause disease in young chickens (Aldous and Alexander, 2008).

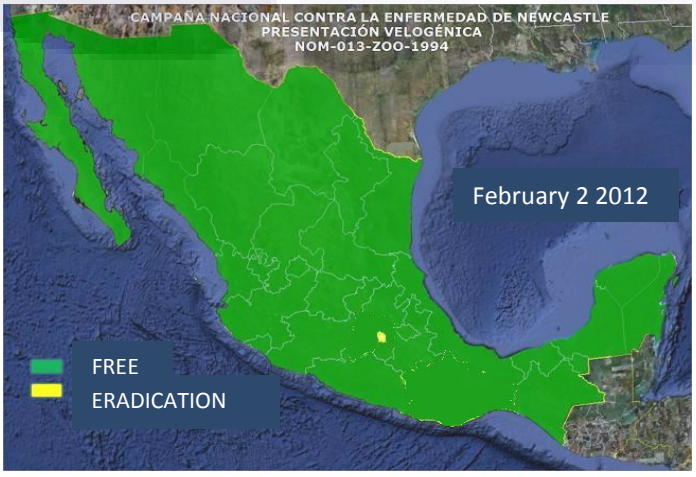


Figure 1.1 Current status of occurrence of velogenic Newcastle disease (vND) in Mexico. **Eradication**= specific geographic area, where zoosanitary conditions take

place toward to elimination of vND or epizootic testings are performed for two years (México city); **Free**= specific geographic area, where there is not been detected positive cases of vND (Rest of states). NOM-013-ZOO-1994. Modified from http://www.una.org.mx/index.php?option=com_content&view=article&id=177&Itemid=115

1.1.2 Clinical and pathology features

The vast majority of references on NDV in poultry are related to chickens, as this species is the most seriously impacted by NDV (Alexander 2003). There is such widely varying disease forms that clinical findings for this species are further divided according to pathotypes. However, the severity of clinical signs does not vary only accordingly to the inherent virulence of the virus, but also according to some host-related factors. These factors are mainly age, route of infection, immune status, and concomitant environmental stress. According to the terrestrial manual (OIE 2009), strains of NDV have been grouped into five pathotypes on the basis of the clinical signs seen in infected chickens. 1) Velogenic viscerotropic: mortality can easily reach 100%, and in experimental conditions, the course of disease is rapid, usually 2–4 days. Clinical signs are first recognizable starting at 2 days postinfection (Brown et al 1999; Kommers et al 2003; Wakamatsu et al 2006). The main signs are conjunctival swelling and reddening centered over the lymphoid patch located in the lower eyelid (Figure 1.2A-D); 2) Velogenic neurotropic: Morbidity with this pathotype often reaches 100%, and mortality is usually 50% (but can rise to 100% in young chickens). The most prominent clinical signs are neurologic and consist of head twitch, tremors, opisthotonus, and paralysis (Figure 1.2E) (Alexander 2003; Brown et al 1999) as well as the lesions are more prominent in the cerebellum, especially within the molecular layer, where they first appear around 5 days postinfection (Figure 1.2F) (Wilczynski et al 1977); 3) Mesogenic: a form that presents with respiratory signs, occasional nervous signs, but low mortality; 4) Lentogenic or respiratory: a form that presents with mild or subclinical respiratory infection; 5) Asymptomatic enteric: a form that usually consists of a subclinical enteric infection.

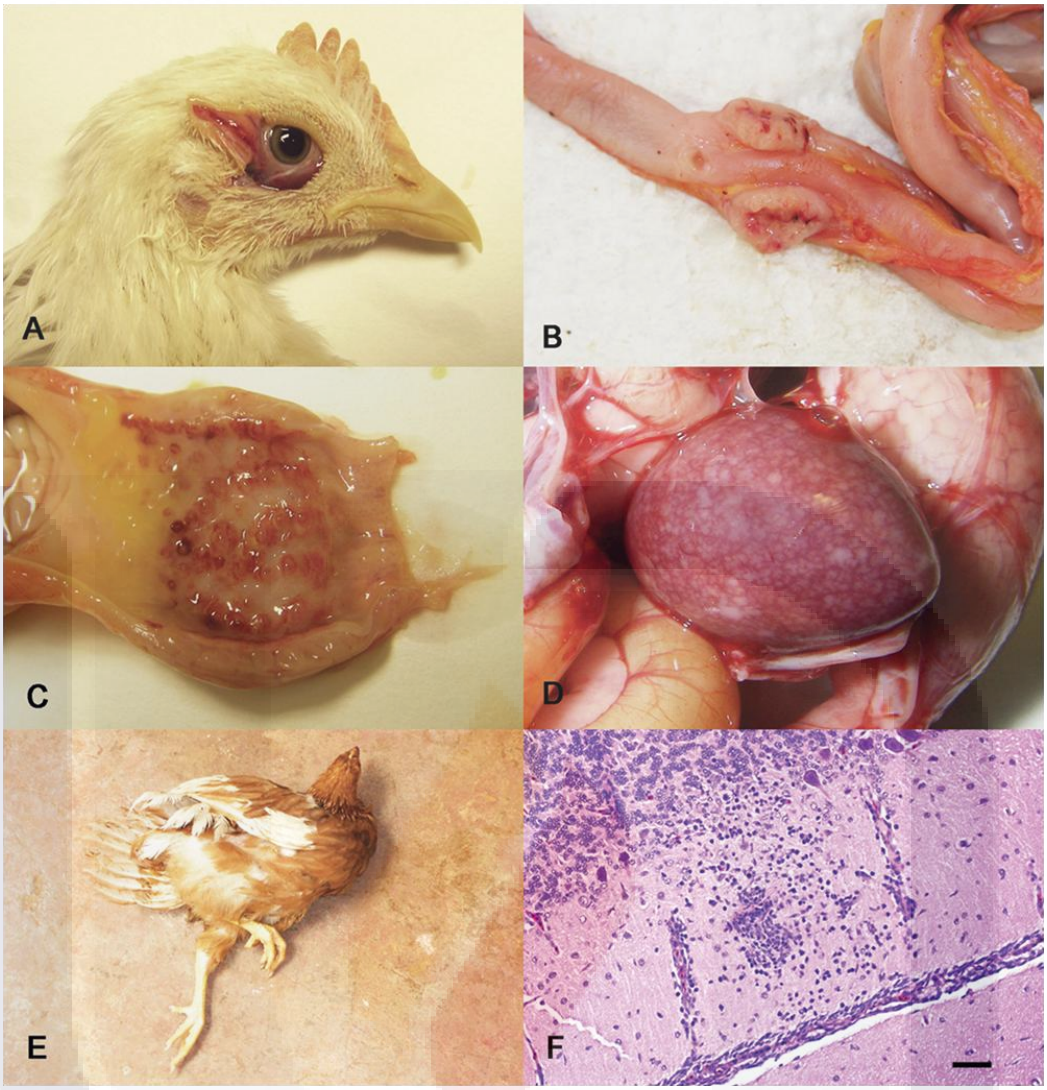


Figure 1.2 Clinical and pathologic features of Newcastle disease virus (NDV). *Velogenic viscerotropic*: **A**, hemorrhage within the crescent-shaped lymphoid patch in the lower eyelid is a characteristic early feature of NDV. **B**, focal hemorrhage and necrosis of cecal tonsils occurs in infection. **C**, hemorrhagic foci in the proventriculus correspond to necrosis of underlying lymphoid tissue. **D**, mottled spleen indicating multifocal necrosis. *Velogenic neurotropic*: **E**, birds are often bright and alert but have hemiparesis. **F**, histologically, brain lesions are prominent in velogenic neurotropic ND and consist of extensive gliosis and astrocytosis; cerebellum. Hematoxylin and eosin. Bar = 100 µm. Modified from Cattoli et al (2011).

1.1.3 Taxonomy and classification

The paramyxovirus family includes multiple viruses that are of importance to global economics and human health. Among the members of the family are well-known, highly infectious worldwide human pathogens such as measles (MeV), mumps (MuV), and respiratory syncytial virus (RSV), a recently discovered human respiratory virus that is also of global significance (human metapneumovirus, HMPV), and deadly zoonotic viruses such as Hendra (HeV) and Nipah (NiV). Paramyxoviruses also cause

disease in other species (such as parainfluenza virus 5 [PIV5] and Sendai virus [SeV]), some of which bring about a tremendous economic burden to society by causing serious, and sometimes fatal, disease in poultry (Newcastle disease virus [NDV] and avian metapneumovirus [AMPV]), cattle (bovine RSV [BRSV]), horses (HeV), and pigs (NiV). While these viruses share many common characteristics, such as possessing a negative-sense single-stranded RNA genome and a lipid bilayer envelope, there are also many unique aspects in their lifecycles. Based on morphologic criteria, the activity of their proteins, and sequence homology, viruses in this family are divided into seven distinct genera, of which five belong to the paramyxovirinae subfamily (Figure 1.3 green) and the remaining two are grouped in the pneumovirinae subfamily (Figure 1.3 red) (Le Bayon et al 2012; Chang and Dutch 2012).

Newcastle disease is caused by viruses of the avian paramyxovirus serotype 1 (APMV-1). These viruses, called APMV-1 virus or NDV, are members of the genus *Avulavirus,* sub-family *Paramyxovirinae*, family *Paramyxoviridae*, order *Mononegavirales.* (Aldous et al 2003; Le Bayon et al 2012 [Figure 1.3]). APMV-1 strains maintained in populations of pigeon have some antigenic differences with other NDV strains that are sometimes called pigeon paramyxovirus serotype 1 (PPMV-1). APMV-1 strains are classified into three pathotypes based on their virulence in chickens. Lentogenic strains are less virulent, the mesogenic are moderately virulent, and the most virulent are velogenic. Most strains are grouped into the extremes of virulence and are either lentogenic or velogenic. Velogenic viruses can be divided into neurotropic form, which is typically associated with respiratory and neurological signs, and a viscerotropic associated with intestinal lesions bleeding (CFSPH 2008; OIE 2009).

Several tests are used to evaluate the virulence of a strain APMV-1 and countries may use different criteria to identify the Newcastle disease. The OIE (Office International des Epizooties) defines it as an infection caused by APMV-1 virus, a highly virulent strain that has either 1) an intracerebral pathogenicity index (ICPI) of at least 0.7 in chickens one day of age, or 2) an amino acid sequence that resembles those observed in highly virulent virus (multiple basic amino acids at the C-terminus of the F2 protein and phenylalanine at residue 117 of protein F1). The US defines "exotic Newcastle disease" as that caused by viscerotropic and velogenic strains. Two different classification schemes for NDV are used to group isolates based on genetic analysis (Miller et al 2010; Kim et al 2007; Seal et al 2005; Liu et al 2011; Aldous et al 2003). Differences in groupings arise between the two classification methods and either can

be used based on preference. One classification proposed by Aldous et al. (2003) is based on genotypes or genetic lineages grouped under serotype 1 (APMV-1). This grouping scheme divides NDV into six lineages (lineages 1 to 6) (Figure 1.4 blue). A second classification method based on the genomic characterization and sequence analysis of the F and L genes groups isolates into either Class I or Class II (Figure 1.4) as opposed to lineages (Kim et al 2008; Miller et al 2010; Kim et al 2007; Liu et al 2011; Liu et al 2009; Perozo et al 2008b). Isolates from Class I are present in the US live bird markets, domestic poultry, and wild waterfowl. Class I is composed of primarily low virulent isolates, but one virulent isolate has been included in that classification. Class I viruses have a worldwide distribution and are further divided into nine genotypes. Isolates grouped in Class I have the longest APMV-1 genome at 15,198 nucleotides. Class I isolates are not usually reported to OIE due to their low virulence designation. Isolates causing all four panzootics from 1920 to the present are classified as Class II (Liu et al 2011). Class II viruses are usually recovered from poultry, pet birds, and wild waterfowl. Class II viruses are further divided into genotypes I through IX (Figure 1.4 red). Genotypes I through IV and IX have slightly shorter genome lengths at 15,186 nucleotides. These genotypes are considered “early” due to their identification between 1930 and 1960. Genotypes V through VIII have a medium length genome at 15,192 and are considered “late” due to their identification after 1960 (Hines and Miller 2012). All velogenic NDV are classified as Class II except for one isolate which caused the Australian outbreak from 1998 to 2000. This isolate was determined to originate from a low virulent strain of NDV which increased in pathogenicity after circulating through poultry (Kim et al 2008; Miller et al 2010; Liu et al 2011). This may explain the classification in Class I where all other isolates are low virulent NDV (Hines and Miller 2012).

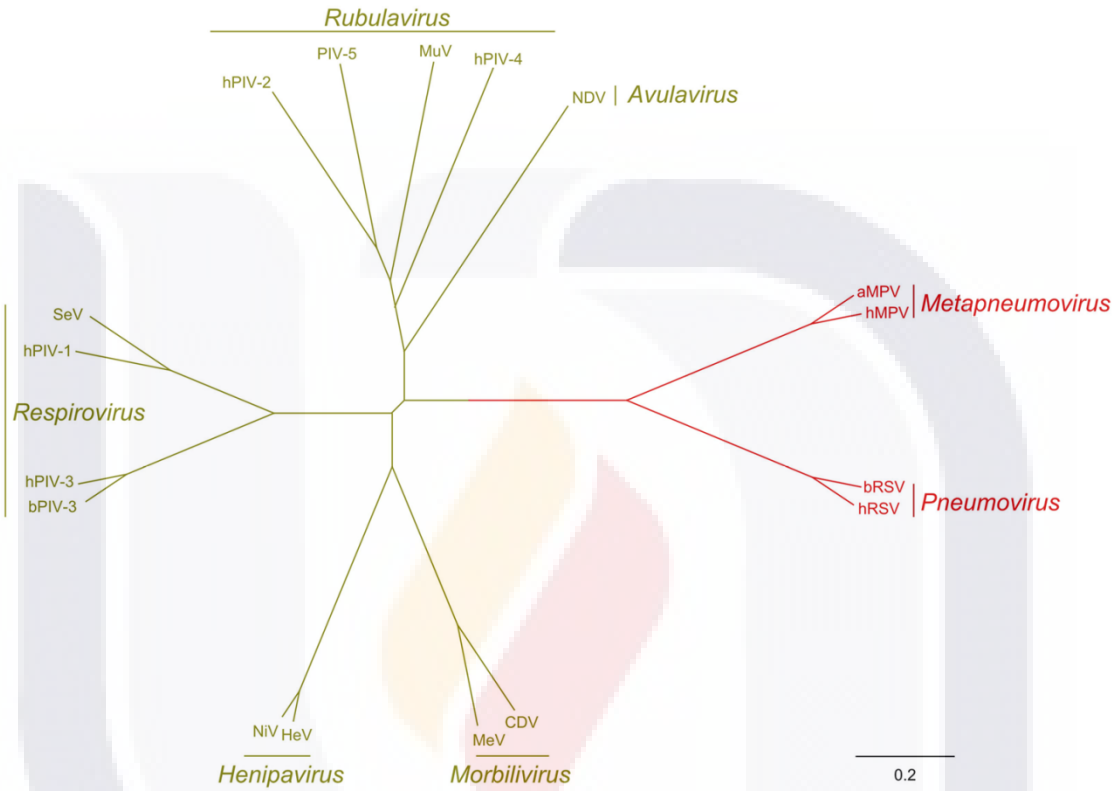


Figure 1.3 Phylogenetic tree of representative members of the *Paramyxoviridae* family. The *Paramyxovirinae* sub-family (green) includes human parainfluenza virus type 2 (hPIV-2), parainfluenza virus type 5 (PIV-5), mumps virus (MuV), human parainfluenza virus type 4 (hPIV-4), Newcastle disease virus (NDV), Sendai virus (SeV), human parainfluenza type 1 (hPIV-1), human parainfluenza type 2 (hPIV-3), bovine parainfluenza type 3 (bPIV-3), Nipah Virus (NiV), Hendra virus (HeV), canine distemper virus (CDV) and measles virus (MeV); the Pneumovirinae sub-family (red) includes avian metapneumovirus (aMPV), human metapneumovirus (hMPV), bovine respiratory syncytial virus (bRSV) and human respiratory syncytial virus (hRSV). Genetic analysis was based on F protein sequence. Modified from Le Bayon et al (2012).

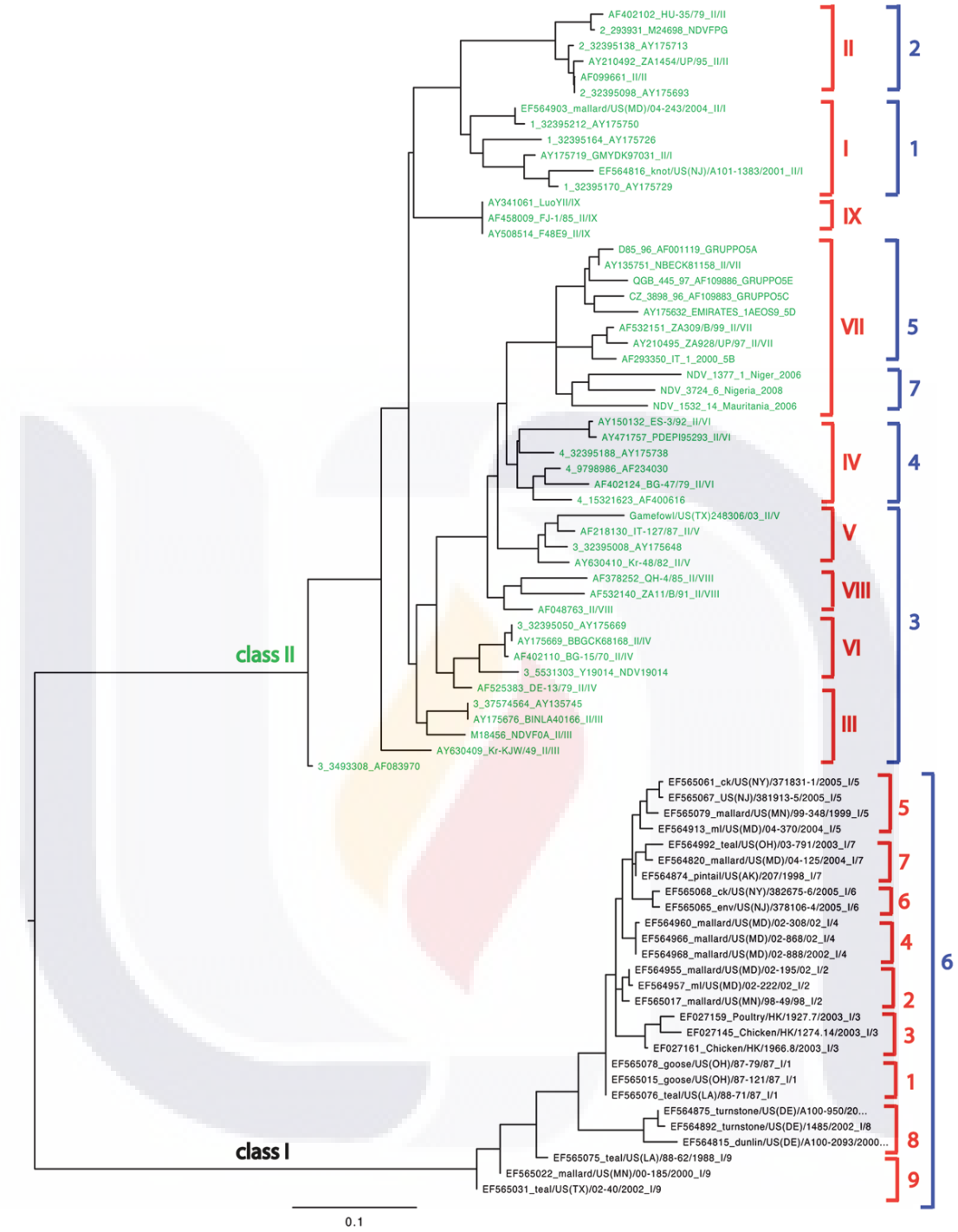


Figure 1.4 Phylogenetic tree for avian paramyxovirus-1, demonstrating 2 distinct classes (I and II) with each having several genotypes. The nomenclatures of the 2 main genotyping systems are indicated in red (Miller et al 2010; Herczeg et al 1999) and in blue (Aldous et al 2003). Modified from Cattoli et al (2011).

1.1.4 Molecular features

As the most of *Paramyxoviridae* family members, the NDV has a genome of single-stranded RNA with a size around 15,186, 15,192 and 15,198 nucleotides, non-segmented, negative polarity protected by helical capsid symmetry, and a sheath

lipoproteic presented in electron micrographs, a pattern of projections of 80 Angstroms length, and the location of antigenic components that give the serological specificity. Its genome codes for at least six proteins including nucleoprotein (N), phosphoprotein (P), matrix (M) protein, fusion (F) protein, hemagglutinin-neuraminidase (HN) protein and RNA polymerase (L) (Figure 1.5). The virion is composed of a stable nucleocapsid core consisting of the NP protein bound to the genomic and antigenomic RNA (Seal et al 2005; Knipe and Hetsley 2001; Zhao and Peeters 2003; Flint et al 2007). The P and L proteins bind to the nucleocapsid core shortly after synthesis to form the ribonucleoprotein (RNP) complex. This RNP complex becomes the template for transcription by the RNA-dependent RNA polymerase L protein. The L protein binds the genomic RNA at a 3' entry site in the RNP complex and transcribes the six protein genes using a start-stop mechanism. In this mechanism the L protein initiates transcription and releases the RNP complex after transcribing a number of nucleotides along the gene which for the *Paramyxoviridae* family is always equal to some multiple of six nucleotides. This transcription requirement is referred to as the "rule of six" (Seal et al 2005; Zhao and Peeters 2003).

The viral particles measured 120 to 180 nm and its envelope glycoproteins have identified two and seven polypeptides. The completeness of the NDV has an average molecular weight of 500 x 106 Daltons, with a density in sucrose of 1.18-1.20 g/ml. (Alexander 1991; Beard and Hanson 1988; Czeglédi et al 2006; de Leeuw and Peeters 1999; May 2002). The two surface glycoproteins, fusion (F) and hemagglutinin-neuraminidase (HN) are important targets of host immune response. Antibodies against the F protein appear to be important to prevent infection and spread of the virus *in vivo*. The major antigenic determinants and epitopes that stimulate the production of virus-neutralizing antibodies have been determined for both the F (Toyoda et al 1988) and HN (Chambers et al 1988) protein.

In fact, there are serological tests to identify the NDV such as hemagglutination and hemagglutination inhibition tests, based in the ability of hemagglutinin to agglutinate chicken´s erythrocytes and some other animal species. In the hemagglutination the virus is adsorbed to the cell receptors causing erythrocyte agglutination, with subsequent avoidance, due to the enzymatic digestion of cell receptor by viral neuraminidase. The time at which the hemagglutinin of the virus is destroyed by heat, is characteristic of each strain of NDV and is a property that can be used to differentiate between one strains from others. The virus has a hemolysin that allows it

to produce varying degree of hemolysis in red blood cells hemagglutinated. Its hemolytic activity is promoted by processes such as freezing, thawing and dialysis (Beard and Hanson 1988; OIE 2009).

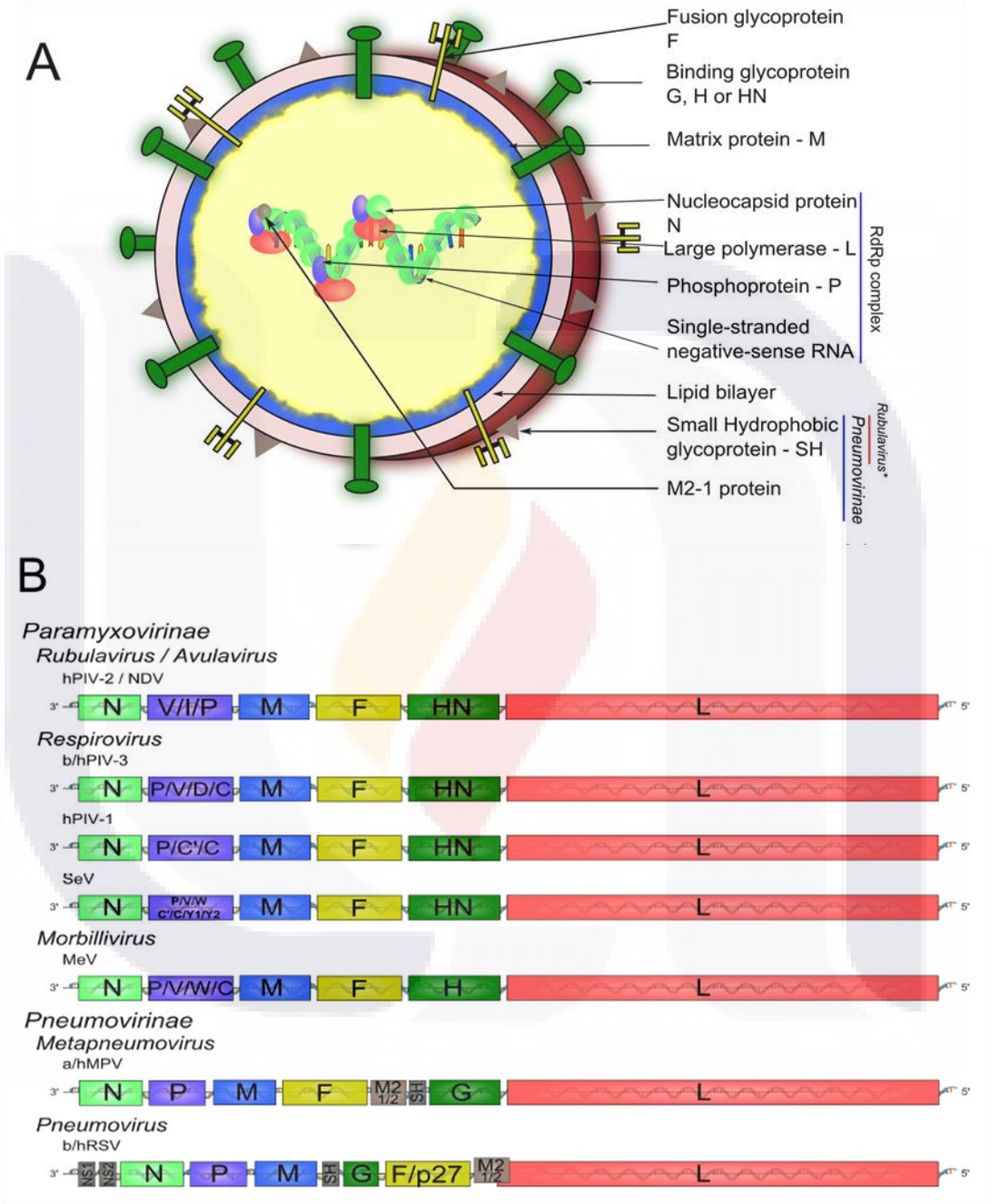


Figure 1.5 Molecular composition of paramyxoviruses. **A,** Schematic representation of a paramyxovirus virion.* The SH protein is present in members of the *Pneumovirinae* and some Rubulaviruses (such as PIV-5 and MuV). **B,** Schematic representation of the genomic organization of selected paramyxoviruses. In *Paramyxovirinae* members, accessory proteins V, I, D, C, C', W, Y1 or Y2 are translated from ORFs and mRNA editing of the P gene. Some proteins are specific to *Pneumovirinae* members such as M2-1 or M2-2, and more specifically to Pneumoviruses, such as the non-structural proteins 1 and 2 (NS1 and NS2). Not to scale. Modified from Le Bayon et al (2012).

1.1.5 Viral Entry into Cells

Viruses have evolved a variety of mechanisms to gain access to host cells and ensure their survival despite the complex protective machinery implemented by the host. In general, after receptor binding, enveloped viruses enter the target cell either by receptor-mediated endocytosis or through direct penetration at the plasma membrane (Chang and Dutch 2012). Most paramyxoviruses and retroviruses have pH-independent fusion proteins, therefore they have been thought to enter cells at the plasma membrane, where the pH is neutral (Figure 1.6A) (Lamb and Jardetzky 2007; Bissonnette et al 2006). This hypothesis is substantiated by the ability of their fusion proteins to promote syncytium formation when expressed at the cell surface under neutral pH and by infectivity studies in the presence of agents that prevent the acidification of endosomes (bafilomycin and ammonium chloride among others) (Mas et al 2011; Srinivasakumar et al 1991). However, direct evidence of viral entry at the cell surface has not been obtained. Indeed, low pH does not inhibit the activity of the fusion proteins of paramyxoviruses like PIV5 (Bissonnette et al 2006), RSV (Srinivasakumar et al 1991), NDV (San Roman et al 1999; Cantin et al 2007), and pH-independent strains of HMPV (Mas et al 2011). Furthermore, RSV and NDV fusion, as assessed by a R18 dequenching assay, is enhanced in acidic environments (Srinivasakumar et al 1991; San Roman et al 1999; Cantin et al 2007). Therefore, the pH requirement for fusion does not necessarily clarify the location of the fusion reaction. Recent studies suggest a more complex mechanism of cell entry for paramyxoviruses. Image correlation spectroscopy studies showed that SeV fusion can occur in the plasma membrane or in intracellular membranes (Rasmusson et al 1998). Other studies using chemical inhibitors, microscopy, and RNAi-mediated knockdown of proteins involved in endocytosis have shown that multiple paramyxoviruses (Chang and Dutch 2012) could at least be partially using endocytic pathways to establish infection (Figure 1.6B). NDV infection was significantly inhibited by agents that sequester cholesterol, and NDV particles were found to colocalize with early endosomal markers, suggesting that NDV may be using the caveolae-dependent endocytic pathway (Cantin et al 2007). Despite being largely insensitive to traditional lysosomotropic agents such as bafilomycin A1 and ammonium chloride, RSV infection was significantly decreased when clathrin light chain, AP1B1, dynamin 3, and Rab5A among others players of the clathrin-mediated endocytosis pathway were knocked down (Kolokoltsov et al 2007). Disruptions of the cellular endocytic and macropinocytic pathways through chemical inhibitors and the expression of dominant negative proteins have been shown to inhibit NiV infection (Figure 1.6C) (Diederich et al 2008; Pernet et

al 2009). However, more studies are needed to determine the exact entry pathway for most paramyxoviruses (Chang and Dutch 2012).

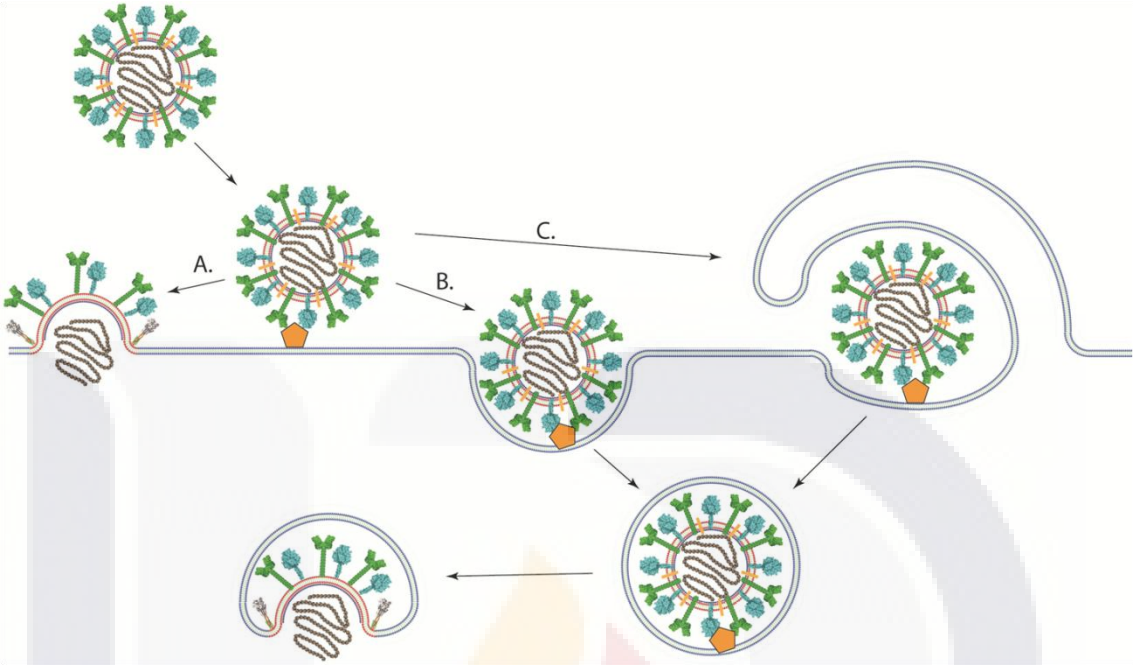


Figure 1.6 Schematic of different pathways of viral entry. **A**, Viral-cell membrane fusion of pH-independent viruses could occur at the plasma membrane under neutral pH after binding to receptor. **B,** A viral particle could enter the cell through an endocytic pathway after receptor binding. Viral-cell membrane fusion could then occur in an intracellular compartment. Low pH may or may not be required for membrane fusion to occur. **C,** viruses could enter cells through macropinocytosis, where the viral particle is engulfed by the cell after receptor binding. Fusion between the viral and cellular membranes would then take place at an intracellular compartment (Modified from Chang and Dutch 2012).

1.1.6 Pathogenesis

The pathogenicity of the virus depends on multiple factors including host species, age, immune status, secondary infections, stress, environmental conditions, the amount of virus transmitted, and the route of transmission but most importantly the strain of the infecting virus (Alexander et al 2004; Saif et al 2008). Chickens are more susceptible than other species, while ducks tend to show no clinical symptoms; thus, waterfowl are considered a natural reservoir for NDV. Cleavage of the F protein during viral replication in the host plays a major role in the virulence of the virus (Alexander et al 2004; Saif et al 2008; de Leeuw et al 2003; Morrison 2001; DiNapoli et al 2009). Velogenic and mesogenic strains of NDV are able to replicate systemically due to the active state of the F protein. Unfortunately velogenic NDV and mesogenic NDV, strains cannot be differentiated based on their amino acid sequences at the F protein cleavage site. Due to the lack of multiple basic amino acids in low virulent strains, the F protein

must be cleaved by secretory trypsin-like proteases which are limited to the mucosal membranes in the respiratory and gastrointestinal tracts. Low virulent strains are not able to replicate systemically due to the limited availability of these trypsin-like proteases (Hines and Miller 2012).

The length of the HN protein has been shown to influence pathogenicity as well (Farkas et al 2009; Zanetti et al 2008). The HN_0 precursor protein is composed of 616 amino acid residues in avirulent strains of NDV including Ulster and D26 (Zanetti et al 2008). This inactive HN_0 is converted to an active protein by proteolytic cleavage of a few nucleotides at the C-terminus. The open reading frame of other NDV strains includes stop codons upstream resulting in active proteins of 571 and 577 amino acids in length. Shortening of the HN active protein plays some role in virulence but is not completely understood (Hines and Miller 2012).

Upon infection with NDV, macrophages of the immune system of chickens produce type I and type II interferon (IFN) (Seal et al 2000). Ten genes encode chicken type I IFN (ChIFN1) while only one gene is responsible for chicken type II IFN (ChIFN2). NDV is able to replicate in these macrophages despite the immune system response. Peripheral blood lymphocytes and heterophils induce apoptosis when infected with the virus. Macrophages of the respiratory system of turkeys infected with NDV show reduction in phagocytic and bacteriocidal abilities (Seal et al 2000). Natural immune stimulation in poultry may not be sufficient to control the disease depending on the infecting strain. Control strategies are needed to prevent development of severe disease (Hines and Miller 2012).

1.1.7 NDV attachment proteins

The ND virion contains two types of surface glycoproteins, the fusion (F) protein and the hemagglutinin-neuraminidase (HN) protein (Saif et al 2008; Knipe and Hetsley 2001; Zanetti et al 2008). The F protein is a class I fusion glycoprotein which is synthesized as a type I integral membrane protein. When the protein is translated, three identical polypeptide chains assemble into homotrimers. Carbohydrate chains are posttranslationally added to the homotrimers which are biologically inactive. Host proteases must cleave the precursor protein in order for it to become biologically active.

The second surface glycoprotein is the HN (Figure 1.5A) protein plays multiple roles in viral entry and egress, including binding to sialic acid receptors, activating the F protein to activate membrane fusion and viral entry, and cleaving sialic acid (neuraminidase) from carbohydrate chains as well as it is able to elicit the immune system (Hines and Miller 2012; Yuan et al 2011). Also it commonly has five N-glycosilation sites located in 120, 341, 433, 481, and 538 possition (Seal 2004). The HN attachment protein is a type II membrane proteins, with N-terminal transmembrane domains (TM) followed by a stalk region and a C-terminal globular head domain (Lamb and Parks 2007). The HN attachment proteins are thought to form tetramers in their active form and are found in a subset of the paramyxoviruses, including parainfluenza virus 5 (PIV5), mumps virus, NDV, Sendai virus, and human parainfluenza viruses (Lamb and Parks 2007; Lamb and Jardetzky 2007; Smith et al 2009; Crennell et al 2000). The C-terminal neuraminidase (NA) domain, obtained by proteolytic cleavage or expression of the NA domain alone, contains the receptor binding site and neuraminidase activity (Crennell et al 2000; Takimoto et al 2000; Yuan et al 2005 [Figure 1.7]).

The HN protein stalk domain carries specificity determinants for F-protein activation, affects neuraminidase activity, and contributes significantly to the oligomerization of the protein (Lamb and Parks 2007; Smith et al 2009; Iorio et al 2009). Mutational studies of the NDV HN stalk have examined effects on membrane fusion, NA activity, hemadsorption, F-protein complex formation, and oligomerization (Stone-Hulslander and Morrison 1999; Gravel and Morrison 2003; Melanson and Iorio 2004; Melanson and Iorio 2006). Although mutations in the NDV HN stalk can affect both NA and membrane fusion activities, it has not been clear how these two functions are coupled. Currently, a four-helix bundle (4HB) stalk packed between two NDV NA domain dimmers has been revealed (Yuan et al 2011), which provide insight into the structural basis for stalk-dependent HN NA and membrane fusion-promoting activities (Figure 1.7).

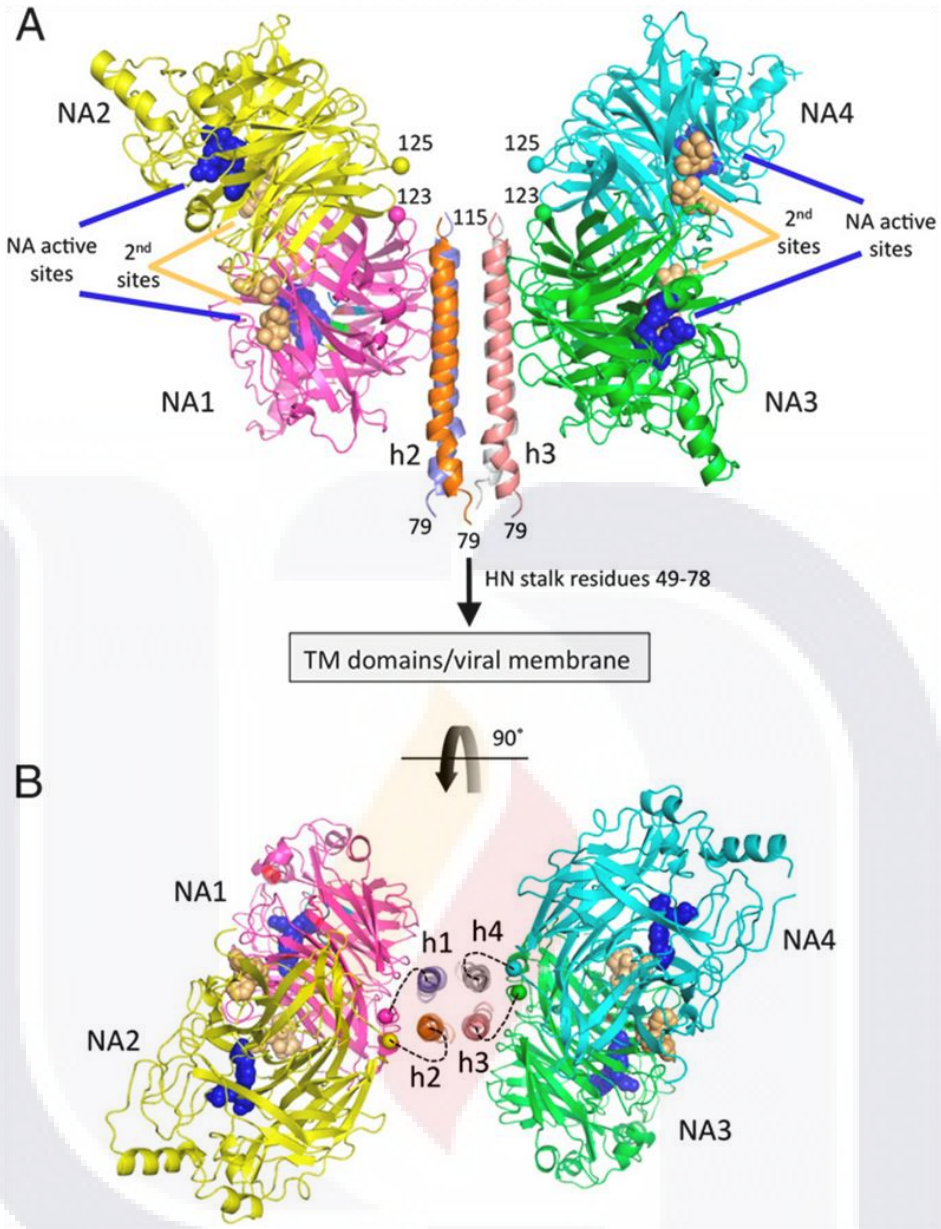


Figure 1.7 Structure of the NDV HN ectodomaim. **A,** Two dimers of the NDV HN NA domains flank the 4HB in the stalk. The four NA domains are labeled NA1–NA4. The active sites are marked by three residues shown as blue CPK spheres (E400, R415, and Y525) and labeled accordingly. The secondary sialic acid binding sites located at the NA domain dimer interfaces are marked by residues shown as orange CPK spheres and labeled (second sites). The N-termini of the four NA domains, residues 123 and 125, are labeled and indicated by their CA atoms shown in CPK format colored by chain. The connections of the N-terminal region of the stalk to the HN TM domains and viral membrane are indicated. **B,** End-on view of the packing of the HN stalk tetramer between two NA domain dimers rotated through 90° as indicated by the curved arrow. Although no electron density was observed to connect the HN stalk helices with the individual NA domains, the dotted lines indicate possible linkages between these domains, with NA1/NA2 and NA3/NA4 forming covalently linked dimmers through C123 and C92 in the S92C mutant. The four-stalk helices are indicated as h1–h4. Modified from Yuan et al (2011).

1.1.8 Transmission

The primary route of transmission is either by ingestion of fecal contaminated material or inhalation of droplets containing the organism (Alexander et al 2004; Saif et al 2008; Seal et al 2000; Li et al 2009). Viral replication in the respiratory tract of infected birds allows for dissemination of the virus during nasal discharge. When the virus reaches the mucous membranes of susceptible birds, the virus is likely to reach the upper respiratory tract. Replication in the respiratory tract of newly infected birds allows for the potential to expose more susceptible birds and the virus easily spreads through the flock. The success of this mode of transmission hinges on the environment temperature and humidity and the viral load contained in the aerosolized droplets. Outbreaks in England from 1970 to 1971 and Northern Ireland in 1973 were attributed to respiratory inhalation of contaminated droplets. The virus is also able to replicate in the intestinal tract which can then be excreted in the feces. It has been shown that large amounts of virus are commonly excreted in the feces of NDV-infected birds.

Several methods of virus transmission have been linked to the introduction of NDV to new premises. Direct ingestion of feed or water contaminated with feces delivers a high virus load to susceptible birds (Alexander et al 2004; Saif et al 2008; Li et al 2009). The virus particles have been shown to enter the eggshell after it has been laid which gives rise to the potential for virus spread during transport of table or hatching eggs. Live or attenuated vaccines may also be a source of infection if the virus used to prepare the vaccine is not properly killed or the vaccine is contaminated. Vaccination and insemination crews as well as veterinarians have been shown to transmit the disease from farm to farm due to improper cleaning and disinfecting of equipment (Hines and Miller 2012). Moreover, migratory wild birds have been shown to transmit NDV to free range poultry through direct contact or by contamination of feed or water (Alexander et al 2004; Saif et al 2008)

Therefore, biosecurity of commercial poultry facilities is an important step in preventing transmission of NDV and large economic loss. It is recommended that poultry farms and hatcheries should not be in close proximity to each other to protect highly susceptible young birds. Separation of farms based on species is important to prevent introduction of exotic diseases to new avian species. The water supply should be clean and should not come from surface water where migratory birds have the potential to contaminate the water source. (Hines and Miller 2012).

1.1.9 Public health

Velogenic strains of APMV-1 can cause conjunctivitis in humans, usually when the person has been exposed to large amounts of virus. Mild self-limiting disease, like flu, with fever, headache and malaise have also been reported in humans, in some cases, it is unknown whether the disease was caused by APMV-1 or wrongly diagnosed by cross-reactions in serological tests. A recent report confirmed by virus isolation, suggests that APMV-1 can cause serious infections in immuno-suppressed people (CFSPH 2008).

1.1.10 Prevention of Newcastle disease

The practice of vaccination for the prevention of animal disease has been used for centuries and has proven to be a powerful tool for the alleviation of animal suffering as well as the economic well being of producers of animal products. Up until 15–20 years ago, vaccines had changed little from those originally pioneered by Jenner and Pasteur. Since that time there have been significant changes in the types of vaccines available owing to a number of factors, including compatibility with eradication programs and international trade policies as well as cost-effectiveness of production (OIE 2010). The associated evolution of new technology in the field of molecular biology and immunology has furthermore had a large impact on the development of new vaccine strategies and the quality of the products that are produced. Some of these strategies are enlisted below:

Reverse genetics. The technology of reverse genetics involves the generation of a cloned copy of complementary DNA (cDNA) from RNA by reverse transcription in vitro, manipulating DNA in vitro followed by generating the modified live virus by transfection of permissive cells with the cloned DNA(s) (OIE 2010). This novel technique has also been used to develop a modified porcine respiratory and reproductive syndrome virus, which can be used as a DIVA (differentiating infected and vaccinated animals) vaccine to help differentiate between vaccinated and infected pigs (de Lima et al 2008). Disabled infectious single-cycle (DISC) vaccine involve the deletion of an open reading frame coding for a key protein involved in the viral replication or viral capsid formation (Widman et al 2008). The DISC virus is isolated in cells expressing the key protein, thus providing the missing protein in *trans*. Such virus, when injected in animals, can complete only one round of replication without producing a progeny virus. Vaccines based on DISC viruses are more stimulatory than a killed virus vaccine and are devoid of problems associated with live vaccines (OIE 2010).

Recombinant Vector Technology. The availability of bacterial and viral genome sequences has facilitated the rapid construction of defined deletions in the genomes of a wide variety of pathogens, which not only results in attenuation, but also creates space for the insertion of foreign genes coding for antigens from heterologous microbes. In general, live bacterial or viral vectors share several characteristics including ease and economy of production, non-integration into the host genome, stability and a reasonable capacity to insert genes coding for heterologous antigens. In addition, like with any live vaccine, the vector should be avirulent and the impact of immunity to the vector should be evaluated (OIE 2010).

Gene-deleted vaccines. The knowledge of specific virulence factor(s) of a pathogen and the availability of recombinant DNA technology has facilitated the creation of specific gene-deleted pathogens for use as live vaccines. The approach of creating and testing defined gene deletions ultimately aids in reducing the pathogenicity/virulence of the organism without affecting the immunogenicity. Such gene-deleted organisms can be used as vaccines as they retain the immunogenic features of the wild-type organism but cannot cause disease. However, to be effective as viable vaccine(s), these organisms should be genetically stable, easy to grow and easy to administer. So far, genes involved either in determining virulence or regulating key metabolic pathways of the organism(s) have been targeted for such deletions (OIE 2010).

Chimeric viruses. Chimeric viruses are defined as recombinant viruses that may contain parts of two closely related viral genomes. For example, a chimeric virus could be one that contains structural genes of one viral serotype and nonstructural genes of another serotype of the same virus. Alternatively, a chimeric virus would be one that contains part of the genome from different members belonging to the same virus family. In principle, chimeric viruses display the biological characteristics of both the parent viruses. One of the main advantages of this approach is that a single dose of chimeric virus delivers the complete repertoire of antigens closely resembling the pathogen(s), which can induce protective immune response against multiple viral pathogens belonging to or different serotypes of the same viral pathogen (OIE 2010).

Subunit vaccines. Subunit vaccines composed of semi-pure or purified proteins have been commercially available since the early 1980s, with subunit components produced by recombinant DNA technology available since the 1990s (Cohen 1993; Rhodes et al 1994; Ulmer et al 1995). The latter have attracted growing interest and activity since

that time. Subunit vaccines do not include live recombinant vector technologies, which provide the delivery of recombinant proteins in vivo. The field of genomics and related areas has revolutionized the manner in which microbial antigens are identified as well as the development of the bioinformatics resources and tools that are required to analyze these genomes has proceeded in parallel and it is now relatively easy to identify surface exposed antigens, specific B- and T-cell epitopes, etc. There is no requirement to have the ability to grow the organism in culture: for example subunit vaccines for *Piscirickettsia salmonis*, a salmonid pathogen, have been developed even though the organism could not be readily grown (Kuzyk et al 2001). The production of subunit antigens can be achieved by both conventional biochemical or recombinant DNA technologies. The latter involves a range of prokaryotic and eukaryotic expression systems including yeast, insect cell and plants (Chichester and Yusibov, 2007) by means of a variety of integrated or transient expression strategies. Subunit vaccines could have some advantages over live attenuated and inactivated vaccines, including the ability to induce strong humoral and cell-mediated immune response. The vaccines furthermore have an excellent safety profile, and can be used in combination with other subunit vaccines. One of the biggest advantages of subunit vaccines is that they are generally compatible with DIVA strategies as long as the antigen is not being used as a marker. However, efficacy is dependent on the protective immunity being induced by inoculation of a single or set of defined recombinant proteins. Experience has shown this may be affected by the gene expression system used. In addition, subunits vaccines may be expensive to produce for some glycoproteins and may require the use of adjuvant to enhance immune responses (OIE 2010).

Virus-like particles. Virus-like particles (VLPs) are supra-molecular structures composed of one or more recombinant proteins. The particles form through self-assembly and typically range from 20 to 100 nm in size. Depending on the origin they can be icosahedral or rod-like in structure. VLPs offer the advantage of formulating the vaccine antigen in a particulate structure, thereby increasing the immunogenicity of the vaccine. VLPs can be used as either vaccine itself or as carrier for genetically fused (chimeric), incorporated or covalently linked antigens (Jennings and Bachmann 2008). VLPs have been extensively studied for the past 20 years, with human vaccines against hepatitis B virus (Zuckerman 2006) and human papillomavirus (Stanley 2008) commercially available and several vaccines for veterinary application in development. These include vaccine for bluetongue virus, rota and parvovirus.

DNA vaccines. DNA vaccines can be defined as antigen-encoding bacterial plasmids that are capable of inducing specific immune responses upon inoculation into a suitable host. Immunization is accomplished by the uptake of purified plasmid in the host cells, where it persists extrachromosomally in the nuclei. Subsequent expression of protein results in the presentation of normally processed or modified forms of the protein to the immune system (OIE 2010). One of the greatest advantages is the ability of DNA vaccines to induce both humoral and cell-mediated immune responses, which is critical for protection from many diseases. However this technology was found to be very effective in rodents, but not performed as well in larger species (Rao et al 2009).

Despite all kind of vaccines describe above, currently only recombinant vectors carrying NDV's epitopes, live and inactivated (included reverse genetic strategy) vaccines against NDV are in the market. Live virus vaccines may be divided into lentogenic and mesogenic groups (Alexander et al 2004; Saif et al 2008; OIE 2008). The immune response has been shown to increase as the pathogenicity of the live virus vaccine increases. To provide the best protection vaccine programs have adopted the method of progressive vaccinations which involves successive booster vaccines with increasingly virulent strains (Alexander et al 2004; Saif et al 2008; OIE 2008; Glisson 2006). Another method begins with low virulent live virus vaccination followed by successive vaccinations using more virulent inactivated viruses (Alexander et al 2004; Saif et al 2008; OIE 2008). This method of combining inactivated and live virus vaccines leads to stimulation of the cell-mediated, innate, and humoral immune responses to improve protection. Live virus vaccines are usually lyophilized allantoic fluid produced by infecting embryonating chicken eggs. The advantages of live vaccines include ease of administration, inexpensive production, and ease of application; however scale-up is limited and a cold-chain is required since production until administration of the vaccine, also cell-mediated immune response initiated by infection by live virus does not offer complete protection against challenge due maternal antibodies (Hines and Miller 2012). Inactivated vaccines are produced using the same method as live virus vaccines, but the virus in the allantoic fluid is inactivated using beta-propiolactone or formalin (Saif et al 2008; Seal et al 2000; OIE 2008). An adjuvant (originally aluminum hydroxide and now oil emulsion) is added to the inactivated virus to stimulate the immune system. Storage of inactivated vaccines is easier than live virus vaccines since the viability of the virus does not have to be maintained. It is labor intensive to produce inactivated vaccines due to the steps required for inactivation and testing to ensure inactivation was complete. Oil emulsion

inactivated vaccines can be used in day-old chicks because the maternal antibodies do not affect the vaccine efficiency (Hines and Miller 2012).

In Mexico, the losses caused by outbreaks in the past were highly significant. In 1975 began the use of inactivated vaccine in Mexico which meant a turning point in the productive results. Therefore, expanded use of emulsified vaccines resulted in excellent results with reduced mortality (Botero 2006). These vaccines confer high levels of protection, by the production of antibodies (mainly IgA and IgG) humoral level. These vaccines do not prevent the spread of the virus, but reduce its replication and protects against mortality of birds (Hines and Miller 2012; Glisson 2006). These emulsified vaccines are widely used in the laying hens to provide protection throughout the production cycle (Al-Garib et al 2003).

1.1.11 Vaccination side effects

Although the vaccinal virus are less invasive than the field virus, they also cause damage to cells of the respiratory tract whether birds are positive for the infection of *Mycoplasma gallisepticum* and *Mycoplasma sinoviae* and the resulting disease can be as severe as that caused by field virus (Jones 2004). It is more common in poultry farms to find vaccinal virus interactions with *M. gallisepticum* and *M. sinoviae*, the disease caused by a virus itself in the field (Jones 2004). Recently in India has been a reported field outbreak after vaccination (3 to 6 days) with the LaSota strain. These outbreaks had increased mortality, decreased food consumption and production. It suggests that there is great similarity between the viral genomes of ND, infectious bronchitis (IB) and the Medium Pathogenic Avian Influenza (MPAI); proteins of the last two can interact with proteins of the virus vaccine (LaSota) increase their virulence, causing disease (Vegad et al 2008). This is the reason why moderately pathogenic vaccinal virus as LaSota has a high incidence of chronic respiratory disease (CRD) as a result of vaccination (Jones 2004). This coupled with the fact that the birds may be colonized by any other bacterial genus such as *Ovnithobacterium rhinotracheale, Bordetella avium* and *Gallibacterium anatis biovar haemolytica* (*Pasteurella haemolytica*), are probably experiencing more severe reactions and develop the CRD after vaccination with live virus (Jones 2004).

On the other hand, in ND the influx of leukocytes in the lumen of the respiratory tract has not been studied, but the evidence has been obtained that suggests that it may have a negative effect on these cells. It has been shown that phagocytes respiratory

birds vaccinated with the NDV have lower phagocytic activity and therefore less bactericidal activity, which explains the appearance in the field of post-vaccine reactions that are caused by concurrent bacterial infections as *M. gallisepticum and M. synoviae* (Al-Garib et al 2003). It is also known that the production of interferon, triggered by the presence of NDV, interferes with replication and spread viral. When the virus overcomes the innate response is probable that triggers an antibody and T cell response. These infiltrations stimulate the necessary elements for the induction of cellular immune response such as macrophages and T lymphocytes such as CD4 and CD8 (Al-Garib et al 2003).

1.1.12 Importance of NDV strains in vaccine formulation

Vaccination continues to be the most important and cost-effective way to control animal and human infectious disease. Although vaccine technology has made substantial progress, the basic concept remains the same. The majority of licensed animal vaccines against virus or bacteria are either live-attenuated or inactivated. Most of the currently vaccines used are composed of either inactivated or live lentogenic strains of NDV. However, live vaccines are infectious and the dead vaccines may induce fears of disease emergence (Gallili and Ben-Nathan 1998). This problem can be overcome if a vaccine consists of only one protein that stimulates the immune response in the host.

To date, NDV surface glycoproteins (F and/or HN) have been the target of many studies for their expression as subunit vaccine in different expression systems such as displayed epitopes in cucumber mosaic virus (Zhao and Hammond 2005; Natilla et al 2006), recombinant vector-based (Perozo et al 2008a), baculovirus-based (Lee et al 2008; Zoth et al 2009; 2011), prokaryotic-based (Lee et al 2010), plant-based either stable (Berinstein et al 2005; Hahn et al 2007; Guerrero-Andrade et al 2006; Yang et al 2007) or transient expression (Gomez et al 2009), plant cell culture (US patent application US2008/0076177, Cardineau et al), and microalgae-based systems (US patent application US2011/0195480, Bayne et al). The most works mentioned above use as antigen either F or HN proteins or, at least their epitopes from LaSota strain (Berinstein et al 2005; Zhao and Hammond 2005; Natilla et al 2006; Perozo et al 2008a; Lee et al 2008). LaSota strain is lentogenic and is classified under the class II genotype II (Miller et al 2010). Also it has phylogenetic clustering with other genotype II strains including BC, Texas GB, B1 and VG/GA (Paldurai 2010), which are the most used either live or inactivated vaccines (USDA 2011). However, the majority of virulent ND strains isolated in North America since 1970 from poultry, psittacines and wild birds

like cormorants and anhingas have been class II genotype V viruses that show nucleotide similarities to the Mexican isolates of 1996 and 1998 (Pedersen 2004). Therefore, if there were to be another outbreak in the US, the etiological agent would likely be a virulent virus similar to the class II genotype V viruses of the recent past and not virulent viruses of the class II genotype II isolates like Texas GB that have not been isolated in the US since the early 1970s (Miller et al 2007).

In order to demonstrate antigenic differences between NDV genotypes, Miller et al (2007) prepared vaccines from ND viruses corresponding to five different genotypes. They were compared to determine if the phylogenetic distance between vaccine and challenge strain influences the protection induced and the amount of challenge virus shed. They showed that Gamefowl/CA/212676/02 strain (CA02, GenBank access number EF520717) that belongs to class II genotype V, has a great potential as a vaccine to protect against strains that belong to other genotypes, it even had better results inducing the production of antibodies in chickens vaccinated with it and then challenged with heterologous antigens. Indeed, one of the causes of vaccine breakdown can be explained by antigenic variation of NDV (Cho et al 2008). Therefore, is important to note that CA02 strain has a mutation in two of three linear epitopes described previously by Iorio et al (1991). Those mutations are I352V and S521T. Currently, Cho et al (2008) demonstrated the importance of the variation in the HN protein's linear epitopes from the Korea strains. They synthesized three oligopeptides from the linear epitope (346-358 amino acids) region. Two of them had one or two mutations (E347K; E347K and M354K) and the other was the common epitope sequence. They found that anti-LaSota chicken antiserum reacts less strongly to oligopeptides with mutations than with the common epitope sequence. Hence, the CA02 strain may be considered as a strong candidate to produce a wide range plant-made Newcastle disease vaccine.

1.2 PRODUCTION OF ANTIGENS IN PLANTS

1.2.1 Plant-made vaccines

Vaccination is the most effective method to control and prevent ND in poultry. Although live attenuated and inactivated whole virus vaccines have been used successfully, both types of vaccines have shown serious drawbacks as former have been reported to cause respiratory distress under certain conditions and there is a risk of reversion to virulent strains with passage from bird to bird (Alexander 2001), whereas mineral oil

present in latter generally results in local inflammation at the site of injection and there is a possibility of contamination by carcinogenic aromatic hydrocarbons (Droual et al 1990; Yamanaka et al 1993). Recent outbreaks of ND underscore the need for continuous evaluation of ND vaccines and vaccination programs (Kapczynski and King 2005).

Given all issues related with current vaccines production methods, since the last century, thanks to genetic engineering, transgenic plants are an emerging technology for the production of recombinant pharmaceutical proteins with many unique advantages. It has already been demonstrated that plants can be used to generate a variety of complex foreign molecules (Moffat 1995; Ma et al 2003). For example, they are effective for the production of recombinant proteins and antigens (Fischer et al 2004; Joensuu et al 2008) and there are already several plant-produced proteins on the market (Howard 2004), including a large-scale (Woodard et al 2003). The concept-proof has been well established for the production of a wide range of therapeutic proteins, including veterinary vaccines (Table 1.1) (Streatfield et al 2003; Dus Santos and Wigdorovitz 2005; Joensuu et al 2008). A key advantage is that plants are higher eukaryotic organisms that possess an endomembrane system and secretory pathways similar to that of mammalian cells, with the advantage of do not propagate animal pathogens. Therefore, complex proteins are generally folded efficiently and assembled with appropriate post-translational modifications (Obregon et al 2006). One of the most obvious benefits of plants is the potential for scale-up. Virtually limitless amounts of recombinant protein could be grown either in contained glasshouses or in the open field. Plant systems would be at least as economical as industrial facilities using fermentation or bioreactor systems and the basic agricultural infrastructure is readily available. However, even though production scale-up can readily be achieved by increasing acreage, it is still necessary to achieve a level of recombinant protein expression (1% of total soluble plant protein (Kusnadi et al 1998)) that is compatible with the required purification technologies appropriate for the application and minimizes the need to handle plant material in bulk (Obregon et al 2006). Even though this issues, plant-based production systems offer several economical, environmental and safety features over other systems, such as described in table 1.1.

Table 1.1 Comparison of recombinant protein production in different systems. *Residual viral sequences, oncogenes, endotoxins; **Large, expensive fermenters; RT, room temperature. Modified from Goldstain and Thomas (2004); Tiwari et al (2009).

	Transgenic plants	Transient expression in plants	Plant cell culture	Yeast	Bacteria	Insect cell/ Baculovirus	Mammalian cell culture
Cost/Storage	Cheap/ RT	Cheap/ -20ºC	Cheap/ -20ºC	Cheap/ -20ºC	Cheap/ -20ºC	Cheap/ -20ºC	Expensive
Dsitribution	Easy	Easy	Feasible	Feasible	Feasible	Feasible	Difficult
Gene size	Not limited	Limited	Not limited	Unknown	Unknown	Limited	Limited
Glycosilation	Correct?	Correct?	Correct?	Incorrect	Absent	Correct?	Correct
Production cost	Low	Low	High	Medium	Medium	High	High
Production scale	Worldwide	Worldwide	Limited	Limited	Limited	Limited	Limited
Propagation	Easy	Feasible	Feasible	Easy	Easy	Feasible	Hard
Protein folding accuracy	High?	High?	High?	Medium	Low	High	High
Protein homogeneity	High?	Medium	High?	Medium	Low	Medium	Medium
Protein yield	High	Very high	Medium	High	Medium	Medium/High	Medium/High
Safety	High	High	High	Unknown	Low	Medium	Medium
Scale-up costs**	Low	Low	High	High	High	High	High
Therapeutic risk*	Unknown	Unknown	Unknown	Unknown	Yes	Yes	Yes
Time required	High	Low	High	Medium	Low	Medium	High
Horizontal gene transfer	Yes	No	No	No	No	No	No

Although a wide range of plant hosts have been developed, non-food crop species like tobacco (*Nicotiana spp.*) are an attractive option for recombinant protein production because they minimize regulatory barriers by eliminating the risk of entry into the food chain. The leaves are harvested before flowering, significantly reducing the potencial for gene leakage into the environment through pollen or seed dispersal. Unlike seeds or tubers, tobacco leaves are perishable and will not persist in the environment. Therefore, tobacco is now recognize as the platform of choice for biopharmaceutical production and is the most common plant species used for the production of subunit vaccine (Table 1.2 and 1.3) (Joensuu et al 2008; Tiwari et al 2009). Moreover, under field conditions, tobacco can produce over 50,000 kg/ha of fresh biomass in a single season (Woodlief et al 1981). However, for oral administration of vaccine antigens in intact leaf tissue, certainly a low cost approach, and the presence of nicotine alkaloids

could limit the use of tobacco but low-nicotine tobacco platforms that are suitable for direct oral administration have been developed (Menassa et al 2007).

Nicotiana benthamiana, in particular, has been a workhorse for studies of plant–virus interactions due to its susceptibility to a wide variety of viruses and ease of use in laboratory settings (Goodin et al 2008). Based on the detailed understanding of viral replication and protein expression obtained from decades of research from many groups, transient expression using viral vectors has become a major strategy for expressing proteins in plants, with the majority of current efforts focused on *N. benthamiana* and tobacco mosaic virus (TMV)-based vector systems (Lico et al 2008; Gleba et al 2007; Smith et al 2006; Pogue et al 2002). Transient expression in *N. benthamiana* offers the advantages of high expression levels, relatively short production times of days to several weeks, and ease of use in controlled growth conditions where optimal parameters for biomass production under good manufacturing practice (GMP) can be obtained. Nevertheless, as has been observed in all prokaryotic and eukaryotic expression systems, there is significant variability in plant expression levels of specific proteins and not all proteins express well in plants. Moreover, within the arena of plant-based expression, a given protein may express much better in one system versus another (Vancanneyt et al 2009). However, one clear conclusion that can be drawn from these studies is that plant-based expression is a viable alternative for the production of some pharmaceutical proteins.

Table 1.2 Subunit vaccine candidates for animal infectious disease control expressed by transgenic plants, plant cell cultures or plant viruses. (a) Only heterologous signal peptides are reported; (b) maximum accumulation level as reported in the literature; 2x p35S, 35S promoter with double enhancer region; 3', polyadenylation signals and site; 5', 5' untranslated region; AlMV, alfalfa mosaic virus; CMV, cucumber mosaic virus; CP, coat protein; CPMV cowpea mosaic virus; FW, fresh weight; GT1, rice glutelin 1; ND, not detected; nos, *Agrobacterium* nopaline synthase; NR, not reported; Ω, untranslated 5' leader from TMV; ocs, *Agrobacterium* octopine synthase; p, promoter; PVX potato virus x; (SE)K/HDEL, endoplasmic reticulum retain signal; SP, signal peptide for secreted proteins; TEV, untranslated 5' leader from tobacco etch virus; TMV, tobacco mosaic virus; TSP, total soluble protein; 35S, Cauliflower mosaic virus 35S; UBQ3, *Arabidopsis* ubiquitin 3. Modified from Joensuu et al (2008).

Pathogen/Host	Antigen	Prod. system	Expression system (a)	Yield (b)	Immune response	References
Infectious bursal disease virus/ Chicken	VP2 protein	Arabidopsis leaves	NR	4.8% TSP	Immunogenic and protective in chickens after oral administration	Wu et al. (2004)
Mink enteritis	VP2 epitope	CPMV	Display on	1,200	Immunogenic	Dalsgaard

virus/Minks		vectors in cowpea	viral particles as part of CP	µg/g FW	and protective in minks following parenteral administration	et al. (1997)
Murine hepatitis virus/Mice	Glycoprotein S 5B19 epitope	TMV vectors in tobacco	Display on viral particles as part of CP	NR	Immunogenic and protective in mice following parenteral or nasal administration	Koo et al. (1999)
Newcastle disease virus/Poultry	F and HN surface glycoproteins	Potato leaves	p35S-5'Ω-3'nos	0.06% TSP	Immunogenic in mice following parenteral or oral administration	Berinstein et al. (2005)
	F and HN epitopes	CMV vectors in tobacco	Display on viral particles as part of CP	430 µg/g FW	ND	Zhao and Hammond (2005)
	F and HN epitopes	PVX vectors in tobacco	Display on viral particles as part of CMV CP	NR	ND	Natilla et al. (2006)
	F surface Glycoprotein	Maize seeds	pUbi-3'35S	3% TSP	Immunogenic and protective in chickens following oral delivery	Guerrero-Andrade et al. (2006)
		Rice leaves and seeds	pUbi-3'nos pGT1-3'nos	0.55% TSP	Immunogenic in mice after parenteral delivery	Yang et al. (2007)
	HN surface Glycoprotein	Tobacco leaves	p35S-5'TEV-3'35S	0.069% TSP	Immunogenic in chickens following oral delivery	Hahn et al. (2007)
Peste des petits ruminant virus/ Farmed and wild Animals	Hemaglutinin-neuramidase	Pigeon pea Leaves	p35S-3'nos	NR	ND	Prasad et al. (2004)
Porcine epidemic diarrhea virus/ Swine	Spike protein	Tobacco leaves	NR	20 µg/g FW	Systemic and mucosal antibodies in mice after oral administration	Bae et al. (2003)
		Tobacco leaves	2xp35S-5'Ω-3'nos	2.1% TSP	ND	Kang et al. (2005a, b)
		Potato tubers	p35S-5'Ω-SEKDEL-3'nos	0.1% TSP	ND	Kim et al. (2005)
	COE epitope as fusion with LTB	Rice seeds	pUbi-3'nos	1.3% TSP	ND	Oszvald et al. (2007)

Porcine parvovirus/ Swine	VP2 capsid protein	Tobacco leaves	2x35S-5'AlMV-3'nos	0.3% TSP	Neutralizing antibodies in mice after parenteral administration	Rymerson et al. (2003)

1.2.2 Genetic transformation methods

Genetic engineering techniques can facilitate the transfer of genes from one species to another (Job 2002). In plants, several methods are available for delivering exogenous DNAs into cells. *Agrobacterium*-mediated transformation particle bombardment and electroporation are routinely used to facilitate gene transfer (Rakoczy-Trojanowska 2002). Indirect DNA delivery via *Agrobacterium*-mediated transformation utilizes the unique ability of this bacterium to introduce transgenes into plant cells. This method usually produces a single copy of the transgene and has high transformation efficiency (Iyer et al 2000). Particle bombardment methods involve bombarding cells with DNA-coated gold or tungsten particles. This method is considered to be widely applicable, but the main limitation is fragmentation of the DNA during bombardment. It often results in a higher frequency of inserting multiple gene copies. These events adversely affect the stability of the transgenes. In addition, the transformation efficiency of the method is relatively low (Gao et al 2008; Travella et al 2005).

Members of the genus *Agrobacterium* have the unique, natural ability to conduct horizontal genetic exchange between organisms of different phylogenetic kingdoms. Best known among *Agrobacterium* species is *Agrobacterium tumefaciens*, which causes the disease crown gall on a wide variety of dicotyledonous plants, as well as on some gymnosperms (Kersters and De Ley 1984; Farrand et al 2003). The fundamental mechanism of pathogenesis is the same for each of these species: DNA transfer from the bacterium to the host plant leads to integration and expression of a portion of a large plasmid [Ti- (tumor inducing) or Ri-(root inducing) plasmid] originally extant in the bacterium. The region of DNA which is processed from these large plasmids is termed the T (transferred)-DNA region, and the transferred DNA is termed T-DNA. T-DNA is exported from *Agrobacterium* and enters the eukaryotic cell as a single-strand molecule called the T-strand. T-strands must traverse the host cell cytoplasm and enter the nucleus, where they eventually may integrate into the host genome. Plant species are the natural hosts for T-DNA transfer; however, animal and fungal cells can also participate as recipient hosts under laboratory conditions (Bundock et al 1995; Piers et

al 1996; de Groot et al 1998; Abuodeh et al 2000; Kunik et al 2001; Bulgakov et al 2006; Lacroix et al 2006a, b; Michielse et al 2008).

The size of T-DNA varies with the type of plasmids, but only the ends, called borders, are recognized during the transfer process. These borders are formed by 25bp flanking the T-DNA region as direct repeats. These border sequences direct the transfer polar form, right-left direction, as determined by the orientation of the repeated extreme. This feature allows removing the middle section of T-DNA and replacing it with the sequence of interest, leaving only the borders (Bevan et al 1983; Fraley et al 1983; Herrera-Estrella et al 1983). The *Agrobacterium*-mediated genetic transformation is a multistep process which begins with recognition and sensing of a wounded host cell, which produces small phenolic molecules such as acetosyringone (AS) (Bhattacharya et al 2010), by a virulent *A*. *tumefaciens. A. tumefaciens* deploys a large number of proteins and uses several molecular machines to initiate and execute the early steps of the transformation process, as illustrated in figure 1.8 (Gelvin, 2003; Christie et al., 2005; McCullen and Binns, 2006). Briefly, proteins encoded by the bacterial chromosomal virulence (*chv*) and tumour-inducing plasmid virulence genes (*vir*) mediate recognition of and attachment to the host cell, production of a mobile T-strand-protein complex (T-complex) and its export into the host cell (Figure 1.8). Once inside the host cell cytoplasm, several Vir proteins (VirD2, VirD5, VirE2, VirE3, VirF) (Shimoda et al 1990; Ashby et al 1988) and host factors (VBF and VIP1 defense-related proteins) (Gelvin 2010; Zaltsman et al 2010; Tzfira et al 2001) act together to deliver the T-complex into the host cell nucleus and integrate it into the host cell genome (Figure 1.8). T-complexes enter the cell nucleus by an active mechanism mediated by the nuclear import machinery of the host cell. Because T-complexes are polar structures, their nuclear import is thought to occur in a polar fashion where the VirD2 molecule attached to the 5′ end of the T-strand may initiate the import process (Figure 1.8) (Sheng and Citovsky 1996). That both VirD2 and VirE2 accumulate in the plant cell nucleus (Lacroix et al 2006b) suggests that not only VirD2, but also VirE2 is involved in the T-complex nuclear import. Once inside the nucleus, the T-strand must be delivered to site of its future integration in the host chromatin. While the exact sequence of events that mediate this intranuclear transport and chromatin targeting is still unknown, various plant factors and several molecular mechanisms have been implicated in these concluding steps of the transformation process. Specifically, CAK2M and TATA box-binding protein (TBP) both of which bind VirD2 (Bakó et al 2003), VIP1 which binds VirE2 (Tzfira et al 2001) and core histones which bind VIP1 (Li

et al 2005; Loyter et al 2005) may function in chromatin targeting of the T-complex. CAK2M interacts with the largest subunit of RNA polymerase II, and the latter recruits TBPs not only for transcription, but also for control of transcription coupled DNA repair. Thus, CAK2M and TBP represent the components of the plant transcriptional and DNA repair machineries, and their interaction with VirD2 (Bakó et al 2003) may target the latter and its cognate T-strand and/or the entire T-complex to the host chromatin (Figure 1.8). At least partial uncoating of the T-DNA from its escorting proteins is necessary for exposing the T-strand to the host DNA repair machinery which will complement it to the double-stranded form and integrate the latter into the host genome. Potentially, this is achieved by the targeted proteolysis machinery of the host cell. The first indication of targeted proteolysis involvement in the transformation process came from the studies of VirF, a bacterial host range factor (Regensburg-Tuink and Hooykaas 1993) exported into the host cell (Vergunst et al 2000). T-DNA integration is the last and perhaps the most host dependent step of the transformation process (Tzfira et al 2004). Host factors are required for complementation of the T-strand molecule to doublestranded DNA (dsDNA), for production of DNA breaks in the host genome and for ligation of the T-DNA molecule into these breaks. Recent evidence, however, indicates that double-stranded breaks (DSBs) in the host genome and double-stranded T-DNA intermediates play an important role in the integration process. It is therefore likely that, T-DNA integration involves conversion of the T-strands into double-stranded intermediates which are then directed to naturally occurring DSBs in the host genome for integration (Citovsky et al 2007).

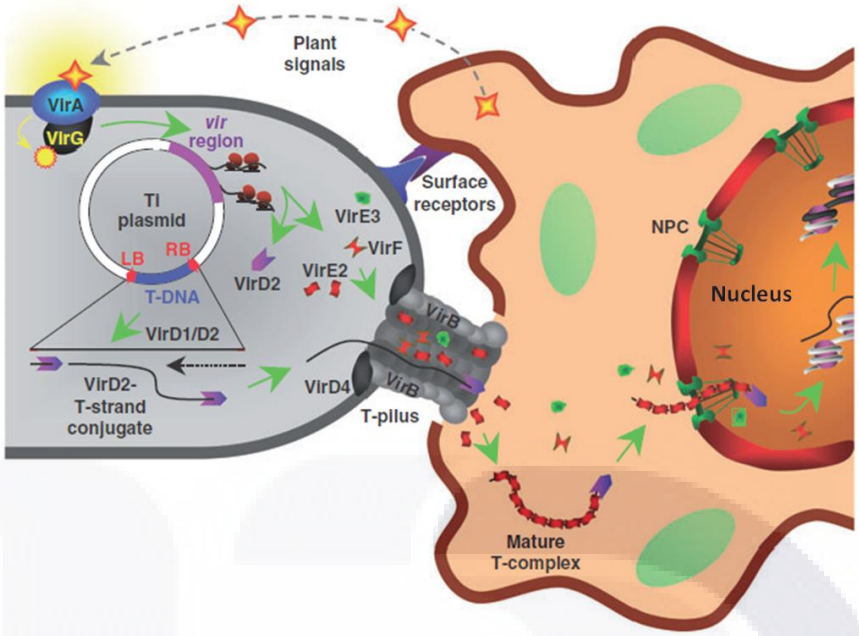


Figure 1.8 Summary of major molecular events and structures within the *Agrobacterium* cell that generate the Vir protein machinery and T-strands which then are transported into the plant cell, enter its nucleus and integrate into the genome. The transformation process begins with recognition of plant signals by the bacterial VirA/VirG sensory system, followed by activation of the *vir* loci and attachment of the bacterium to the host cell. The T-strand is excised from the T-DNA region by VirD2/VirD1 and exported, *in cis* with a covalently attached VirD2 molecule and *in trans* with several other Vir proteins, into the plant cell cytoplasm via a VirB/D4 type IV secretion system. Inside the host cell, the VirD2–T-strand conjugate is packaged by numerous molecules of VirE2 to form a mature T-complex. Then mature T-complex, leaded by VirD2, enters the cell nucleus through nuclear pore complex (NPC) by an active mechanism mediated by the nuclear import machinery of the host cell. Once inside the nucleus, the T-strand must be delivered to site of its future integration in the host chromatin through host and bacterial factors such as VBF/VIP1 and VirF, respectively. Modified from Citovsky et al (2007).

1.2.3 Transient expression and agroinfiltration

The field of plant-made pharmaceuticals (PMPs) has steadily evolved since the expression of a functional mouse IgG in tobacco was published in 1989 (Hiatt et al 1989) to the point where several plant-produced proteins have been used in clinical trials (Aviezer et al 2009; Kaiser 2008; McCormick et al 2008). Having several PMPs in advanced clinical trials is a major breakthrough and has initiated more focused efforts on developing a regulatory process for approving plant-made biologics (Sparrow and Twyman 2009). Along with this, a number of plant-based expression systems have been developed to express recombinant proteins. These expression systems include plant cell cultures and intact plants, and the use of both stable transformation and transient expression systems (Vancanneyt et al 2009). Plant cell culture systems derived from plants that have been used to produce biopharmaceuticals include

tobacco, tomato, soybean, rice, carrot cells, and *Arabidopsis thaliana* (Hellwig et al 2004; Shaaltiel et al 2007; Plasson et al 2009). Other cell-based systems include algae and moss bioreactors (Decker and Reski 2007; 2008; Leon-Banares et al 2004). Even though much of the early work was carried out with stably transformed plants or cells, there has been an increasing trend towards the use of transient expression systems in recent years, mostly in plant-made vaccines (Table 1.3). The major reason for this is sheer convenience and speed: both virus vector-based and *Agrobacterium* infiltration-based systems offer the chance of getting large amounts of protein in days after the initial molecular cloning event, rather than the months necessary for transgenic expression (Fischer et al 1999).

Table 1.3 Transient expression of vaccine antigens in plants. Modified from Tiwari et al (2009).

Plant/Tissue	Vector	Phatogen	Disease	Antigenic protein	Reference
Tobacco/leaf	pTRAc (Agroinfiltration)	HIV-I	AIDS	HIV-1Pr55Gag, Gag (p17/p24), p24	Meyers et al (2008)
Tobacco, Spinach/leaf	AIMV	Rabies virus	Rabies	Chirmeric peptides of rabies glycoprotein (RGP) and rabies nucleoprotein (RNP)	Yusibov et al (2002)
Tobacco/leaf	TMV	Porcine epidemic diarrhea virus	Actue enteritis	PEDV-COE (Core neutralizing epitope of porcine epidemic diarrhea virus)	Kang et al (2004)
Tobacco/leaf	Plum pox potyvirus	Canine parvovirus	Myocarditis and fatal enteritis	Capsid protein VP2	Fernandez - Fernandez et al (1998)
Tobacco/leaf	PVX	Rotavirus	Gastroenteritis	Inner capsid protein	O'Brien et al (2000)
Tobacco/leaf	pCaSF1-V110 (Agroinfiltration)	*Yersinia pestis*	Pneumonic/Bubonic Plague	F1, V and F1-V fusion protein	Mett et al (2007)
Tobacco/leaf	TMV based agroinfiltration	*Mycobacterium tuberculosis*	Tuberculosis	Ag85B, ESAT-6 and ESAT-6: Ag85B fusion	Dorokhov et al (2007)
Tobacco, collard/leaf	pICH115999 (Agro infiltration)	Vaccinia virus	Smallpox	Vaccinia virus B5 coat protein	Golovkin et al (2007)
Tobacco/leaf	TMV based agroinfiltration	*Bacillus anthracis*	Anthrax	LicKM-LFD1	Chichester et al (2007)
Tobacco/leaf	pBID4 (agro infiltration)	Avian influenza virus H5N1	Avian flu	Influenza virus haemagglutinin antigen (HA)	Shoji et al (2009)
Tobacco/leaf	pLKT60 (Agro infiltration)	Shipping fever	Bovine pneumonic Pasteurellosis	*Manheimia haemolytica* A1 leukotoxin 50	Lee et al (2001)

Transient expression systems are practically limited to virus-based (Whole recombinant or deconstructed) and *Agrobacterium*-mediated somatic expression, either in whole plants (Rybicki 2010) or in harvested leaves (Plesha et al 2009; Sudarshana et al 2006). While alleviating environmental and regulatory concerns associated with the production of transgenic plants since transient production can be performed on harvested nontransgenic plant tissue to produce heterologous proteins within a contained facility and eliminate exposure of recombinant material to the environment (Joh and VanderGheynst 2006). In *Nicotiana* plants transient expression of recombinant proteins is currently performed by the use of engineered infectious plant viruses or *Agrobacterium*-mediated DNA transfer (agroinfiltration). Most of these are based on RNA viruses such as tobacco mosaic virus (TMV) and potato virus X (PVX) (Lico et al 2008) (Table 1.3). The first generation plant virus vectors utilize infection-competent viruses, represented by the modified TMV-based Geneware® system (Kentucky BioProcessing, LLC, Owensboro, KY). In essence, such a vector is comprised of the viral cDNA harboring a gene of interest either as a fusion to viral coat proteins (CPs), mainly for epitope presentation as vaccine antigen (Smith et al 2009), or placed downstream of an additional subgenomic promoter (Lico et al 2008; Wagner et al 2004). Viruses are inoculated into the leaf initially as infectious RNA, which is created from the vector either through *in vitro* transcription or agroinfiltration followed by *in planta* transcription (Wagner et al 2004). Thus, the protein of interest is coexpressed along with systemic viral spread and replication, with maximal expression usually obtained within 2–3 weeks postinfection. A recent notable example of recombinant proteins expressed by infectious virus-based systems is the antiviral lectin Griffithsin. Using the Geneware® system, functional Griffithsin was expressed in *N. benthamiana* at a very high level, reaching as high as 5 g of the protein per kg of leaf biomass (O'Keefe et al 2009). Such high levels of expression with this type of virus vectors are, however, usually limited to small proteins whose coding sequences are less than 1.5 kb. This is due to the increased genetic instability of recombinant viruses carrying a larger foreign sequence (Gleba et al 2007; Avesani et al 2007). Despite this limitation, this method offers a viable option for the mass production of small proteins such as antiviral lectins and monoclonal antibody single-chain variable fragments (scFvs).

A major breakthrough in viral expression strategies was facilitated by the recent advent of deconstructed virus vectors, originally reported for the TMV-based magnICON® system, developed by ICON Genetics GmbH (Halle, Germany) (Marillonnet et al 2004).

The essence of improvements in this system from the first generation viral vectors are: (1) deletion of the viral CP gene to enhance the stability and size compatibility of a transgene, (2) viral cDNA modifications facilitating *in planta* RNA replicon recovery upon *Agrobacterium*-mediated DNA transfer, and (3) efficient whole-plant vector delivery by vacuum-based agroinfiltration ("magnifection") to compensate for defective systemic movement due to CP deletion (Gleba et al 2005; Marillonnet et al 2005). These improvements allowed the uniform and high-level (gram per kg biomass) expression of larger proteins in *N. benthamiana* plants within 10 days. ICON Genetics further developed a similar deconstructed viral vector system based on PVX. Taking advantage of the fact that TMV and PVX do not compete during replication, fully assembled immunoglobulin (Ig)G molecules were expressed at up to 0.5 g per kg of leaf by co-delivering deconstructed TMV and PVX vectors (each encoding a gene for Ab heavy or light chains) (Giritch et al 2006). This technology may provide the most rapid means among all currently available recombinant expression systems for the production of full length monoclonal antibodies from genes in various production scales ranging from bench to commercialization (Hiatt and Pauly 2006). A potential limitation of the magnifection method is that it is technically challenging to scale-up; however, this impediment has recently been solved by development of a robotic magnifection system by Kentucky BioProcessing, LLC. Another example of a deconstructed TMV-based system is the "Launch vector", developed by Fraunhofer USA Center for Molecular Biotechnology, Newark, DE. This combines the advantageous features of standard agrobacterial binary plasmids and plant viral vectors, to achieve high-level target antigen expression in plants. As an additional feature, to aid in target expression, stability and purification, a thermostable carrier molecule (lichenase) was engineered to which antigens are fused. This launch vector/carrier system was applied to engineer and express target antigens from various pathogens, including, influenza A/Vietnam/04 (H5N1) virus (Musiychuk et al 2007). In addition, using the TRBO: A High-Efficiency Tobacco Mosaic Virus RNA-Based Overexpression Vector (Lindbo 2007), some foreign proteins such as *Phytopthora infestans* Avr3a, *Aequorea victoria* GFP, *Arabidopsis* adenosine kinase, 10th type III (FN10) domain from human fibronectin, tomato RCR-3 proteinase, and tomato P69b proteinase, were expressed at levels of 3 to 5 mg/g fresh weight of plant tissue in 3-7 days post-infiltration.

Currently a tripartite Cucumber Mosaic Virus viral amplicon (CMVva) expression system has been reported (US Patent application 20120045818-Plant-Based Production of Heterologous Proteins, Hwang et al 2012). The original CMVva system

was developed by mixing the CMV subgroup I amplicon segments RNA 1 and RNA 2 together with CMV subgroup II amplicon segment RNA 3. Segments RNA 1 and RNA 2 encode for replication-associated proteins and the host defense protein 2b, the silencing suppressor. Amplicon segment RNA 3 encodes for the 3a movement protein and the capsid protein. However, the subgroup II RNA 3 segment has been modified for insertion and expression of heterologous genes. To ease its manipulation during cloning, RNAs were inserted in separately plasmid. Using this system has been demonstrated its efficiency expressing in harvested *N. benthamiana* and sunflower leaves of two heterologous proteins such as GFP and an endoglucanase E1 from *Acidothermus cellulolyticus.* Previously Sudarshana et al (2006) showed a Cucumber Mosaic Virus inducible viral amplicon (CMViva) expression system, capable of a high level of transient production of heterologous proteins in leaves of N. benthamiana. Subsequently, the same group (Plesha et al 2007; 2009), showed that the production of biologically functional recombinant α1-antitrypsin (AAT) increased by optimization of the induction process by which the induction solution makes contact with the plant cells. They also showed that high levels in transient production were possible in leaves harvested through a method for applying the inducer that is more benign to the plant leaf.

On the other hand, agroinfiltration with conventional nonviral binary vectors had been primarily used for analytical purposes before constructing transgenic plants (Matoba et al 2004). However, progress made in recent years now allows even these vectors to express proteins at higher levels with agroinfiltration compared to transgenic plants. One of the key factors for high expression with agroinfiltration-delivered nonviral vectors appears to be the coexpression of a viral suppresors of RNA silencing (VSRs) such as tomato busy stunt virus-derived p19 and potyviral helper component proteinase (HC-Pro), which its mechanism will be described more detailed later (Vézina et al 2009; Villani et al 2009; Voinnet et al 2003; Ma et al 2009; Wydro et al 2006; Huang et al 2009; Arzola et al 2011). Additionaly, a series of new highly efficient agroinfiltration expression vectors (pEAQ vectors) has been constructed based on a conventional binary vector containing cauliflower mosaic virus 35S promoter, and modified 5'-UTR and the 3'-UTR from Cowpea mosaic virus RNA-2 within the T-DNA region (Sainsbury et al 2009). These vectors were shown to express multiple polypeptides along with P19 from a single plasmid at a high level within a few days.

According to discussion before of virus-based systems, they seem to be the best choice for heterologous proiteins production. However, since methods of applying virus directly to the plant tissue have typically resulted in poor infection efficiency and therefore low protein yield (Gleba et al 2007) the preferred method to transfer the viral amplicon and target gene into the plant is to use *A. tumefaciens* to deliver T-DNA containing cDNA copies of the modified viral genome to the host plant cells (Tiwari et al 2009). For this reason, a wide variety of agroinfiltration methods have been described in the literature, ranging from injecting a small amount of bacterial solution into the abaxial surface of a plant leaf using a syringe without a needle (Schob et al 1997), to spraying the *A. tumefaciens* solution onto plants with an airbrush with or without surface wounding of the leaf (Azhakanandam et al 2007), to vacuum infiltration of harvested leaves immersed in an *A. tumefaciens* solution (Kapila et al 1997), to injection of fruits with an solution using a needle (Orzaez et al 2006), to vacuum infiltration of the leaves and shoots of intact plants by turning them upside down and immersing them in an *A. tumefaciens* solution and then applying and releasing a vacuum (Gleba et al 2007). Of all the methods currently in use, vacuum infiltration of leaves is the least manually labor intensive and therefore most amenable to scale-up and automation (Plesha et al 2009; Tiwari et al 2009). Vacuum infiltration is a two part process. First, leaf tissue is submerged in the solution of recombinant *A. tumefaciens* and a vacuum is applied, which causes the gasses from the stoma spaces to be drawn out of the leaf; and second, the vacuum is released and the solution is vigorously forced into the cavities of the leaf tissue to equilibrate the pressure difference (Simmons et al 2009). Therefore, combination of virus-based vectors and vacuum agroinfiltration is to date the more promising method to produce plant-made proteins large-scale.

1.2.4 Anriviral RNA silencing and viral silencing RNA suppressors

RNA silencing is a conserved sequence-specific gene regulation system, which has an essential role in the development and maintenance of genome integrity in a wide variety of organisms. In higher plants and insects, RNA silencing also operates as an adaptive inducible antiviral defence mechanism (Ding and Voinnet 2007; Ding 2010). The silencing of RNA relies on host- or virus-derived 21–24 nucleotide long sRNA molecules, which are the key mediators of RNA silencing-related pathways in plants and other eukaryotic organisms (Phillips et al 2007; Voinnet 2009; Ruiz-Ferrer and Voinnet 2009; Llave 2010). In plants, similar to other eukaryotic organisms, there are two main types of sRNAs, miRNAs and siRNAs, but the siRNA class contains several

different types (Vaucheret 2006; Brosnan and Voinnet 2009). These sRNAs are produced from double-stranded RNA (dsRNA) or from folded structures by Dicer-like proteins (DCLs), and they guide Argonaute (AGO) proteins to target cognate RNA or DNA sequences (Ruiz-Ferrer and Voinnet 2009). These endogenous sRNAs play important roles in many aspects of gene regulation in plants, controlling developmental programming or biotic and abiotic stress responses (Ruiz-Ferrer and Voinnet 2009; Mlotshwa et al 2008). Both cellular and antiviral siRNA biogenesis often requires RNA-dependent RNA polymerases (RDRs). However, plant viruses are efficient pathogens, which are able to infect and invade distinct plant species. They often cause severe symptoms and damage, which suggests an efficient counter defensive strategy against the antiviral silencing response. Many viral silencing RNA suppressors (VSRs) have been identified since the discovery of the first VSRs more than a decade ago (Table 1.4) (Anandalakshmi et al 1998; Brigneti et al 1998; Kasschau and Carrington 1998). The various VSRs are able to target all effectors of the silencing pathway, such as viral RNA recognition, dicing, RISC assembly, RNA targeting and amplification (Figure 1.9).

Table 1.4 Plant viral suppressors of RNA silencing. [a]Tospoviruses and tenuiviruses replicate in their insect vectors and in plants. Modified from Roth et al (2004).

Genus	Virus	Suppressor	Evidence	Reference
Carmovirus	Turnip crinkle virus (TCV)	CP	TCV infection does not reverse silencing. In agro-coinfiltration assay, CP blocks sense and antisense induced local silencing and prevents systemic silencing.	Qu et al. (2003) and Thomas et al. (2003)
Closterovirus	Beet yellows virus (BYV)	P21	Suppresses inverted repeat (IR) induced local silencing in agro-coinfiltration assay. BYV p21 corresponds to BYSV p22.	Reed et al. (2003)
	Beet yellow stunt virus (BYSV)	P22		
Cucumovirus	Cucumber mosaic virus (CMV)	2b	Infection with CMV or with PVX-2b vector blocks silencing.	Li et al. (2002),
	Tomato aspermy virus (TAV)		Interferes with systemic signal	
Furovirus	Beet necrotic yellow vein virus (BNYVV)	P14	Agro-coinfiltration assay with sense induced silencing. BNYVV P14 corresponds to PCV P15.	Dunoyer et al. (2002)
Geminivirus	African cassava mosaic virus (ACMV)	AC2	Infection with ACMV, PVX-AC2, or PVX-C2 reverses silencing.	Dong et al. (2003), Voinnet et al. (1999) and van Wezel et al. (2002)
	Tomato yellow leaf curl virus-China (TYLCV-C)	C2	Blocks sense induced silencing in agro-coinfiltration assay. AC2 and C2 are homologs.	
Hordeivirus	Barley stripe mosaic virus (BSMV)	γb	RNA mediated cross protection between PVX-GFP and TMV-GFP vectors is eliminated when γb is expressed from the PVX vector.	Yelina et al. (2002)
	Poa semilatent virus (PSLV)			
Pecluvirus	Peanut clump virus (PCV)	P15	PCV infection blocks silencing. p15 blocks local and delays systemic sense-induced silencing in agro-coinfiltration assay.	Dunoyer et al. (2002)

Polerovirus	Beet western yellows virus (BWYV) Cucurbit aphid-borne yellows virus (CABYV)	PO	BWYV PO suppresses local but not systemic sense-induced silencing in agro-coinfiltration assay. CABYV PO tested only on local silencing.	Pfeffer et al. (2002)
Potexvirus	Potato virus X (PVX)	P25	PVX infection does not suppress silencing. In agro-coinfiltration, p25 blocks systemic but not always local silencing	Roth et al (2004)
Potyvirus	Potato virus Y (PVY) Tobacco etch virus (TEV)	HC-Pro	Evidence from multiple types of assay. Does not block systemic silencing in stable expression grafting assay, but does in agro-coinfiltration assay	
Sobemovirus	Rice yellow mottle virus (RYMV)	P1	Infection with PVX-P1 viral vector reverses silencing.	Voinnet et al. (1999)
Tenuivirus[a]	Rice hoja blanca virus (RHBV)	NS3	Agro-coinfiltration assay of sense induced local silencing.	Bucher et al. (2003)
Tombusvirus	Tomato bushy stunt virus (TBSV) Cymbidium ringspot virus (CymRSV)	P19	Limited activity in reversal of silencing; strong activity in agro-coinfiltration. AMCV (artichoke mottled crinkle virus) P19 also works as a suppressor.	Voinnet et al. (2003), Qu and Morris (2002) and Takeda et al. (2002)
Tospovirus[a]	Tomato spotted wilt virus (TSWV)	NS_s	TSWV infection reverses silencing. In agro-coinfiltration, NSs suppressed sense, but not IR, induced local and systemic silencing.	Bucher et al. (2003) and Takeda et al. (2002)

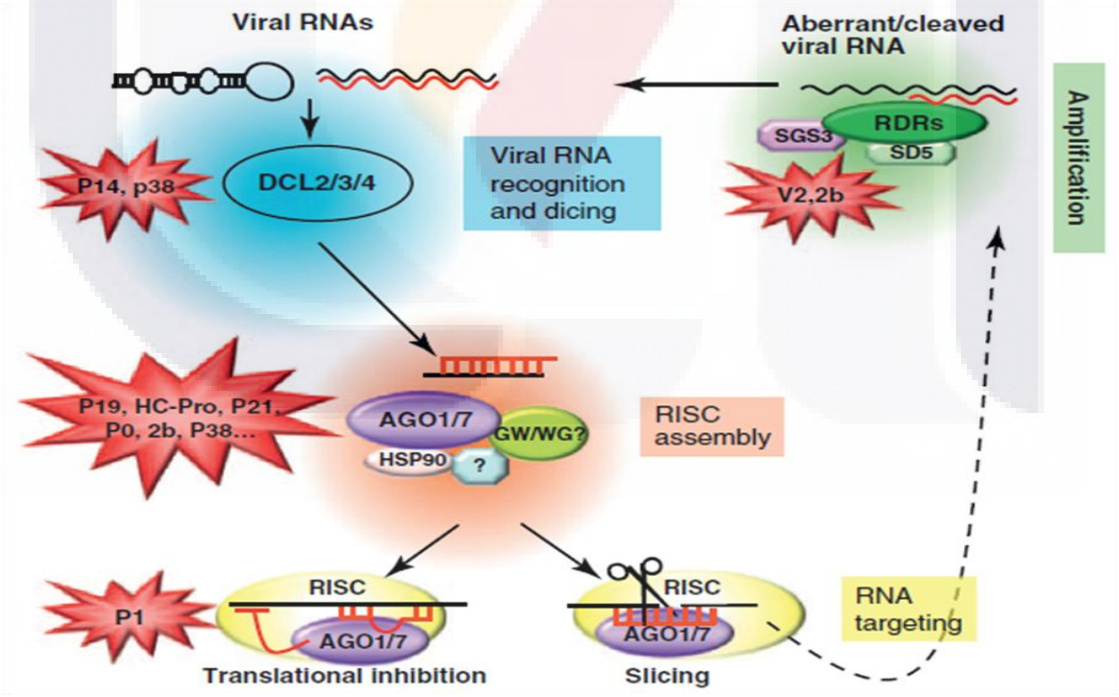


Figure 1.9 Current model of antiviral RNA silencing in plants and its suppression by virus-encoded silencing suppressors. RNA silencing is initiated by the recognition of viral dsRNAs or partially ds hairpin RNAs, which are processed to vsiRNAs by dsRNA-specific RNases called DCLs (DCL2/3/4). In the next step, HSP90-activated AGO1/7 (Iki et al 2010) are loaded with vsiRNA, thereby forming large RISCs, which probably also incorporate other unidentified proteins (e.g. GW/WG motifs containing AGO

interactor proteins). Afterwards, the vsiRNA-loaded RISC targets viral RNAs by slicing or translational arrest. Secondary vsiRNAs are produced in an amplification loop through the actions of RDRs and their cofactors (SGS3 and SD5) (Jauvion et al 2010). Viral-silencing suppressors can disrupt these pathways at multiple points, thereby preventing the assembly of different effectors or inhibiting their actions. The points at which certain VSRs (i.e. P14, P38, V2, 2b, P19, HC-Pro, P21, P0 and P1) interact with the silencing pathways are depicted. Modified from Burgyán and Havelda (2012).

This viral mechanism to avoid plant immunity has biotechnology applications, several researchers have demonstrated that agroinfiltration and CaMV-driven transient protein production in non-transgenic plants can be enhanced if VSRs are used (co-infiltration) to block posttranscriptional gene silencing (PTGS) in the infiltrated leaves. Notable examples of co-infiltration with different VSRs have recently been reported and proving that VSRs are another way to increase plant-made pharmaceuticals expression. Sudarshana *et al* (2006) evaluated whether co-infiltration with *A. tumefaciens* containing the gene *p19* from Tomato bushy stunt virus encoding a VSR, improves the production of recombinant α1-antitrypsin (rAAT) with CMViva expression system. The presence of p19 also increased the percentage of functional rAAT in relation to the level of total rAAT, resulting in a maximum of 70 ± 3.3% in this study. Also, note that the addition of p19 enhanced the expression rAAT although CMViva was designed to encode the CMV 2b silencing suppressor. On the other hand, Vézina et al (2009) reported up to 1.5 g of full-size, assembled IgG was expressed in 1 kg of *N. benthamiana* leaf in 4–6 days by co-expressing the heavy and light chains, HC-Pro, and a chimeric human β1,4-galactosyltransferase (GT) by infiltrating a mixture of four Agrobacterium strains, each delivering either of four constructs. As well, Arzola et al (2011) tested the efficiency of several VSRs through co-infiltration with a recombinant anthrax receptor fusion protein (CMG2-Fc), reaching up to 0.56 g per kg of leaf fresh weight after 3.5 days postinfiltration.

1.2.5 Codon optimization

Other important factors of successful *Agrobacterium*-based transient expression include the strain of *Agrobacterium*, density of the bacteria, infiltration media, infiltration condition, and the plant's physiological condition (Wroblewski et al 2005, Plesha et al 2009), along with vector and transgene design. Latter is important at many different levels of biological research and for biopharmaceuticals production, because of depends in large part upon the protein expression levels that can be achieved. Therefore, genetic constructs for the expression of proteins now frequently use synthetic DNA (Welch et al 2009). This is because sequence information from genome

and metagenome sequencing projects has increased exponentially over the last decade (Venter et al 2004), but most of these sequences are not available as physical DNA. Several biotech companies provide gene synthesis services at an affordable price. Any DNA sequences can be designed and synthesized with a fast turnaround time of less than 1 month. At the present time, the bottom line price has already dropped to about $0.35/base for individual customers. So it currently costs only $500 (US Dollar) to synthesize a typical gene of 1.5 kilo base pairs with sequence confirmation (Jung and McDonald 2011). Moreover, it has a great advantage in that it allows the redesign and optimization of native DNA sequences to improve gene expression. There have been many successful reports demonstrating over-expression after sequence optimization. Most current synthetic gene design strategies are guided by mimicry of natural gene characteristics thought to be relevant for increased expression (Itakura et al 1977). A variation on this approach is to copy the codon bias of a subset of highly-expressed native host genes (Henaut and Danchin 1996) or even to exclusively use the codons most common in highly expressed genes (Fuglsang 2003) maintaining the expression of the same amino acids.

Although it is still controversial and recent work has called into question its usefulness as a predictor of expression in some hosts, CAI (Codon adaptation index) or codon bias has been one of the most commonly used indexes to evaluate genes (Jung and McDonald 2011). According to results from recent large scale experiments, gene expression level varied more than 40 fold in 40 variants and up to 250 folds in 154 variants (Welch et al 2009; Kudla et al 2009). These studies provide compelling evidence that synthetic gene design can have a significant impact (Jung and McDonald 2011). However, the mechanism of gene expression is complicated at the molecular level and codon bias is not the only determinant for gene expression efficiency. For example, there is an active debate on the relationship between codon bias, mRNA folding energy, and gene expression. Moreover, regarding gene design criteria, there are many other factors such as Shine-Dalgarno or Kozak's context sequence, repeated sequences, potential polyadenylation sites, cryptic splice sites, introns, and nuclease cleavage sites as well as restriction enzyme sites, GC content, UTR (untranslated region), and use of rare codons that affect gene expression (Jung and McDonald 2011).

Due to the complicated gene design criteria, gene optimization is not easy since it requires huge repetitive computations. There are several software packages currently

available such as Codon optimizer (Fuglsang 2003), DNAWorks (Hoover and Lubkowski 2002), DyNAVacS (Harish et al 2006), GeMS (Jayaraj et al 2005), Gene Composer (Lorimer et al 2009), Gene Designer (Villalobos et al 2006), GeneDesign (Richardson et al 2010), GeneOptimizer (Raab et al 2010), JCat (Grote et al 2005), OPTIMIZER (Puigbò et al 2007), Synthetic Gene Designer (Wu et al 2006), and UpGene (Gao et al 2004). In order to facilitate gene design, Jung and McDonald (2011) have developed unique gene design software called Visual Gene Developer. The software provides a user-friendly interface and includes many useful functions such as mRNA secondary structure/binding energy prediction, codon usage/mRNA optimization, GC content/Nc (effective number of codons)/CAI calculation, sequence comparison, repeated sequence search, multiple query sequence search, and silent removal of undesirable sequences. All those features make the software a useful tool to redesign and optimized genes foreign to be expressed in any organism.

2. JUSTIFICATION

Newcastle disease (ND), a viral disease of birds with a wide range of clinical signs, is capable of causing economic losses worldwide (Alexander 2001), including Mexico (Botero, 2006). ND is caused by viruses of the avian paramyxovirus serotype 1 (APMV-1) (Aldous et al. 2003). APMV-1 strains or ND virus (NDV) are classified into three pathotypes based on their virulence in chickens. Lentogenic, mesogenic and velogenic, the most virulent (CFSPH 2008; OIE 2009). The virus of Newcastle disease (NDV) has a genome of single-stranded RNA with a size around 15,186, 15,192 and 15,198 nucleotides. Its genome codes for at least six proteins including nucleoprotein (N), phosphoprotein (P), matrix (M) protein, fusion (F) protein, haemagglutinin-neuraminidase (HN) protein and RNA polymerase (L). The surface proteins (F and HN) are important targets of host immune response. In addition, the major antigenic determinants and epitopes that stimulate the production of virus-neutralizing antibodies have been determined for both the F (Toyoda *et al*. 1988) and HN (Chambers *et al*. 1988; Iorio et al 1991) protein.

Therefore, vaccination is the most effective method to control and prevent ND in poultry. Although live attenuated and inactivated whole virus vaccines have been used successfully, both types of vaccines have shown serious drawbacks as former have been reported to cause respiratory distress under certain conditions and there is a risk of reversion to virulent strains with passage from bird to bird (Alexander 2001).

As an alternative, thanks to genetic engineering, transgenic plants are an emerging technology for the production of recombinant pharmaceutical proteins with many unique advantages. It has already been demonstrated that plants can be used for the production of recombinant proteins and antigens (Fischer et al 2004). However, due to the long time that takes to produce stable transgenic plants (months), there has been an increasing trend towards the use of transient expression systems in recent years. The major reason for this is sheer convenience and speed: both virus vector-based and *Agrobacterium* infiltration-based systems offer the chance of getting large amounts of protein in days after the initial molecular cloning event, rather than the months necessary for transgenic expression (Fischer et al 1999).

With this background, this work proposes to generate a vaccine against ND, using as epitope the HN protein sequence through the biomanufacturing platform SwiftVax®, which involves inserting the target gene either in a viral-based (Cucumber Mosaic Virus viral amplicon (pCMVva), Tobacco Mosaic Virus RNA-Based Overexpression (pTRBO)) or non viral-based (p35S) expression system, and vacuum-based agroinfiltration in *Nicotiana benthamiana* leaves. A similar system using a CMV-based inducible viral amplicón (CMViva) has already produced significant expression of a human blood protein, α1-antitrypsin (Plesha *et al*. 2009; Sudarshana *et al*. 2006). Thus, it is likely that the platform SwiftVax® will be effective for the expression of a ND subunit vaccine.

3. HYPOTHESIS

Using the biomanufacturing platform SwiftVax® (transient expression by vacuum-based agroinfiltration) it will be possible to transiently express and recover the NDV HN protein in harvested *Nicotiana benthamiana* leaves.

4. GENERAL OBJECTIVE

To produce and to characterize of the Hemagglutinin-Neuraminidase (HN) protein in harvested tobacco leaves, produced by the biomanufacturing platform SwiftVax®.

4.1 PARTICULAR OBJECTIVES

a) Cloning of genes engineered (HNop 1, HNop2, HNop3 and HNop4) into of at least one of the following binary vectors: pCMVva, p35S or pTRBO.

b) Verify the correct insertion of the genes redesigned into of at least one of the binary vectors mentioned above.
c) Transfer at least one of the binary vectors with the genes designed into *Agrobacterium tumefaciens*.
d) Use recombinant *Agrobacterium tumefaciens* for transient expression in harvested *Nicotiana benthamiana* leaves by vacuum agroinfiltration.
e) Protein extraction and quantification of expression level of HN protein in the agroinfiltrated leaves.

5. METHODOLOGY

5.1 *IN SILICO* DESIGN OF THE HN GENE CONSTRUCTS FOR TRANSIENT EXPRESSION IN *NICOTIANA BENTHAMIANA*.

5.1.1 Selection of NDV strain to express the HN protein.

To produce a poultry vaccine against ND, we used as antigen the hemagglutinin-neuraminidase (HN) glycoprotein, which is part of viral envelope of NDV. The HN protein has been characterized and recognized as the main trigger in the immune response in poultry. Moreover, it has been expressed already in potato and tobacco plants either by stable or transient expression (reviewed by Joensuu et al 2008). Three main epitopes have been described previously (Iorio et al 1991) in different regions of the protein: 193-201, 346-353, and 513-521. Those epitopes are conserved among the different NDV strains. However, punctual mutations in the epitope sequence or around it have likely been the cause of the current outbreaks in some countries (Gu et al 2011; Cho et al 2008). For this reason, to choose which NDV strain we will use, we considered some strains that had been isolated from the last outbreak in North America and that belong to main genotype circling in America, genotype V (Perozo et al 2008b). The strain that we chose was Gamefowl/US(CA)/ 212676/2002 (CA02); it was isolated from the recent outbreak in 2002-03 in California and belongs to genotype V. The HN cDNA sequence was obtained from GenBank (access number EF520717) and in order to determine the punctual mutations into the epitopes, we performed a multiple sequence alignment with different NDV genotypes using ClustalW2 software (http://www.ebi.ac.uk/Tools/msa/clustalw2/). Another reason that influenced our choice for the CA02 strain was that its immunogenic capability has already been verified by challenging CA02-vaccinated chickens with homologous or heterologous NDV strains (Miller et al 2007).

5.1.2 Codon optimization of the HN gene sequence and additional elements.

Once the strain was selected, we engineered the HN cDNA sequence to be transiently expressed in *N. benthamiana* leaves by vacuum agroinfiltration. In order to increase the expression level of the protein, when we engineered it, we considered the following features: 1) Codon optimization, which was performed using Visual Gene Developer software (Jung and McDonald 2011). This software has a *N. benthamiana* codon-usage table, so the codon optimization was made only for the open reading frames (ORF's). 2) Subcellular localization, we chose three different signal peptides for this approach: i) native HN (HN protein's first 48 N-term amino acids) for constructs HNop2 and 4, ii) 2S2 storage albumin from *Arabidopsis thaliana* (MANKLFLVCATFALCFLLTNA) for construct HNop1, and iii) RAmy3D rice alpha-amylase (MKNTSSLCLLLLVVLCSLTCNSGQA) for construct HNop3. 3) Addition of an endoplasmic reticulum (ER) retention sequence "KDEL" (Lys, Asp, Glu and Leu), used widely in the expression of heterologous proteins (Joensuu et al 2008), except in the HNop3 construct which only has a signal peptide (RAmy3D) to lead the protein to the apoplast. 4) As an enhancer untranslated sequence (5'UTR) we used *psaDb* gene sequence, except for HNop4, from *N. sylvestris* (Yamamoto et al 1995), which has already been shown to be an efficient transcription enhancer. 5) The 3xFLAG-tag sequence (DYKDHDGDYKDHDIDYKDDDDK) that has been successfully applied for recombinant protein purification (Einhauer and Jungbauer 2001); this sequence was placed in all the constructs before the 49 N-term amino acid and after the signal peptide. Also added was a multiple cloning site at the 5' (PacI-XhoI-PstI) and 3' (HindIII-SpeI-AvrII) ends to facilitate the cloning into the different binary vectors. Once the genes were optimized and their elements were added, they were sent to be synthesized by an outside company (GeneWiz), and a couple weeks later we received 5µg lyophilized of four different constructs (Figure 6.2) within the commercial vector pUC57 (Figure 5.1).

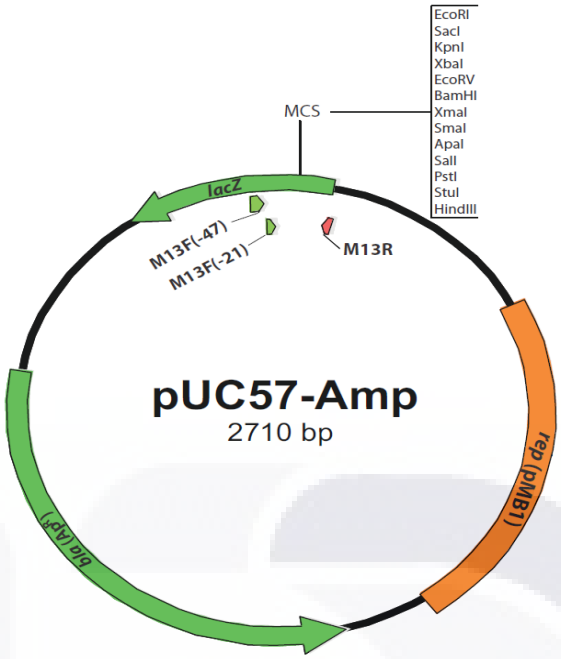


Figure 5.1 Plasmid map of the commercial vector pUC57-Amp without the HNop constructs. The *lacZ'* region (encoding the lac α peptide) with a multiple cloning site (MCS) and the Ampicilin (Ap^R) bacterial selectable marker are indicated by the green arrowed boxes, which denote the orientation of the coding region. Replication origin (pMB1) is indicated by an orange box.

5.2 GROWTH OF *NICOTIANA BENTHAMIANA* PLANTS.

5.2.1 Preparation of *Nicotiana benthamiana* plants.

The plants were grown in the greenhouse at UC Davis as follows. The *N. benthamiana* TW 16 seeds were planted in a 6-inch pot with UC soil mix and grown for 2 weeks, and then the seedlings were carefully removed and transplanted to individual 6-inch pots. Three seedlings were transplanted per pot and were kept growing for additional 3-4 weeks prior agroinfiltration. Growth conditions included a 16-h photoperiod and temperature range of 75-85°F (25-30°C). To ensure optimum health throughout the growth cycle, a drip irrigation system was used and Osmocote fertilizer (Scotts Miracle-Gro Company, Marysville, OH) was added in the irrigation water. All experiments were carried out in upper leaves 5-6 weeks old *N. benthamiana* leaves, harvested immediately before agroinfiltration.

5.3 CLONING OF HNop CONSTRUCTS INTO THE p35S, pCMVva AND pTRBO BINARY VECTORS, VERIFICATION OF CORRECT INSERTION AND TRANSFER INTO *AGROBACTERIUM TUMEFACIENS.*

5.3.1 Transformation of competent cells with pUC57:HNop1-4

All experiments corresponding to cloning work were carried out in Dr. Dandekar's lab (Department of Plant Science, 170 Robbins, UC Davis). In order to manipulate all the constructs synthesized by GENEWIZ, we transformed One Shot® TOP10 Chemically Competent *E. coli* cells (Invitrogen cat#C4040-10) with each one of the four synthesized constructions (HNop1-4) following the company's protocol. Then, to select the transformed cells with the four different constructs, 10µl of each cell culture were plated and spread with glass beads on Luria-Bertani (LB) 1.5% agar medium added with ampicillin 100µg/ml as bacterial marker and incubated at 37ºC overnight. Colonies that grew well were grown in 5ml of liquid LB medium with the same antibiotic added were placed at 37ºC and grown overnight with 225rpm of shaking. Then, to obtain a freezer stock of each construct, inside of the laminar flow hood in a 1.5ml centrifuge tube, 750µl of each cell culture was mixed with 250µl of 60% glycerin and stored at -80ºC until used to clone into the different binary vectors.

5.3.2 Cloning of HNop1-4 constructs into p35S binary vector

The p35S binary vector was formed from two vectors pDE00.0113 and pDU97.1005 (Figure 5.3), both of them kindly supplied by Dr. Dandekar's lab. As described above, One Shot® TOP10 Chemically Competent *E. coli* cells were transformed with each vector, to facilitate its manipulation in the cloning work and freezer stocks were generated as well. To begin with the cloning, freezer stocks of the constructs pUC57:HNop1-4 and pDE00.0113 were cultured in 5ml of LB medium with appropriate antibiotics added (ampicillin 100µg/ml for pUC57:HN1-4 and kanamycin 50µg/ml for pDE00.0113) and incubated at 37ºC overnight with 225 rpm of shaking. Then, 2ml of each cell culture was placed into a 2ml centrifuge tube and spun down for 1min at 13,000 rpm, then the supernatant was decanted and the pellet was process to obtain plasmid deoxyribonucleic acid (DNA) using QIAPREP® Spin MINIprep Kit (cat# 27104). Once the plasmid DNA for each construct was obtained, the DNA concentration was measured using a NanoDrop 1000®. First to free each HNop construct from the pUC57 vector, the plasmid DNA of each one was digested with XhoI and HindIII restriction enzymes, that corresponded to multiple cloning site (MCS) located in the pDE00.0113 (Figure 5.3a), which was also digested with the same enzymes (Annex A, Protocol 1).

The reason why the four constructs were first cloned in the pDE00.0113 vector is because this has the constitutive CaMV (Cauliflower Mosaic Virus) 35S promoter and

its respective terminator (ocs 3') with a MCS between them and they also are flanked with an AscI site (Figure 5.3a), making the cloning easy for each HNop construct with the promoter and terminator in the pDU97.1005 binary vector. Once the plasmid DNA of each HNop construct and the vector were cut with the proper restriction enzymes (XhoI and HindIII), 20µl of each digestion reaction was loaded (except for pDE00.0113 vector's reaction 5µl were loaded) with 2µl of loading buffer in a 0.8% agarose gel and the electrophoresis was performed as in annex A, protocol 2 (Figure 5.2). When the four HNop constructs were able to be isolated from the pUC57 vector, the respective bands (~2kb) were excised from the gel using a sharp scalpel and placed in separated 1.5ml centrifuge tubes. Then, the DNA was recovered from each gel slice using the QIAquick Gel Extraction Kit (cat# 28704). The pDE00.0113 vector was not be recovered, since it was just run to make sure it had been cut properly by the restriction enzymes. After that and in order to insert each HNop construct in the pDE00.0113 vector, a ligation was performed following annex A, protocol 3. Once the ligation was made, One Shot® TOP10 Chemically Competent *E. coli* cells were transformed, following the company's protocol, with each HNop construct previously ligated into the pDE00.0113 vector.

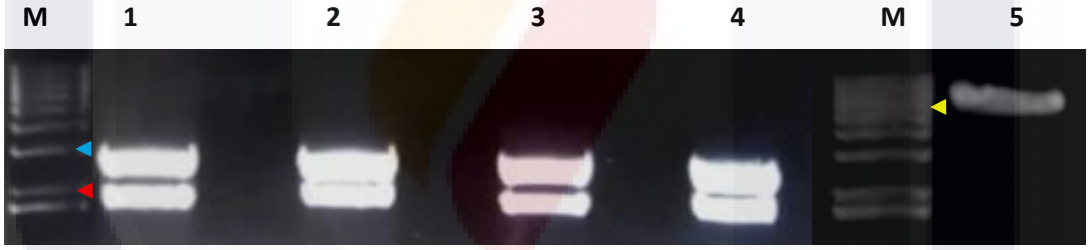


Figure 5.2 Plasmid DNA of pUC57:HNop1-4 and pDE00.0113 digested with XhoI and HindIII restriction enzymes. A 0.8% agarose gel was used to visualize and recover bands of HNop1-4 constructs (~2kb). M) 1 kb Plus DNA Ladder (Invitrogen cat#10787-018), 1) HNop1 (1.779kb), 2) HNop2 (1.860kb), 3) HNop3 (1.791 kb), 4) HNop4 (1.837kb), and 5) pDE00.0113 vector (6.137kb). Red, blue and yellow arrows stand for 2kb, 3kb and 6kb respectively.

Then, to select the transformed cells with the four different vectors generated (pDE:HNop1, pDE:HNop2, pDE:HNop3, and pDE:HNop4), 10µl of each cell culture were plated and spread with glass beads on LB 1.5% agar medium with kanamycin added at 50µg/ml as the selective agent and incubated at 37ºC overnight. After that, positive colonies were selected through *colony PCR* (Annex A, Protocol 4). This protocol is to identify rapidly and easily, which colonies have been successfully transformed with the pDE:HNop1-4 vector. Appropriate primers (Table 5.1) were used to amplify from 90nt upstream of the CaMV 35S promoter end, until a little more than

the middle of each HNop sequence, to obtain a PCR product of ~1kb, which means that the HNop construct was successfully ligated within the pDE00.0113 vector and is present into the colony.

When at least a positive colony with each pDE:HNop vector was identified by colony PCR, they were cultured in 5ml of LB medium with kanamycin added at 50µl/ml and grown at 37ºC overnight. Also a pDU97.1005 freezer stock was cultured in the same conditions, except that gentamicin at 20µl/ml was added instead of kanamycin. After that, the plasmid DNA of each cell culture was obtained as previously described using QIAPREP® Spin MINIprep Kit (cat# 27104) and the concentration measured with the NanoDrop 1000®. In order to clone each pDE:HNop vector into the pDU97.1005 binary vector, all the vectors were digested with the AcsI restriction enzyme as shown in annex A protocol 2, since this site is present in the MCS of the pDU97.1005 binary vector (Figure 5.3b) and the 35S promoter and the ocs3' terminator are flanked with this site (Figure 5.3a). Once the pDE:HNop vectors were digested with the AscI restriction enzyme, the size of the pDE00.0113 backbone (~4kb, without the 35S pro and ocs3' ter) and the size of each pro-HNop insert (~4kb, HNop construct plus the 35S pro and ocs3' ter) were almost the same, so it was impossible to excise only the band that corresponded to the pro-HNop insert. To overcome this issue, first the pDU97.1005 binary vector was dephosphorylated as shown in annex A, protocol 5. After this procedure, the probability of the pDU97.1005 binary vector to be religated to itself was very low, only leaving the opportunity of ligation either to the pDE00.0113 backbone or the pro-HNop inserts. Second, the digestion made with AscI for each pDE:HNop vector was not recovered from a gel, it was used directly in the ligation reaction (Annex A, Protocol 3 with modifications: 2µl of the pro-HNop insert were added instead of 1µl of ddWater, resulting in a 2:1 proportion insert and vector) with the dephosphorylated pDU97.1005 binary vector. However, to make sure all the digestion worked well, prior to the ligation 3µl of all the digestions were loaded with 2µl of loading buffer in a 0.8% agarose gel and electrophoresis was performed as in annex A, protocol 2.

After the ligation reaction between the dephosphorylated pDU97.1005 binary vector and each pro-HNop insert (pro-HNop1, pro-HNop2, pro-HNop3 or pro-HNop4), One Shot® DH5α-TI^{R} Chemically Competent *E. coli* cells were transformed following the company's protocol. Then, 10µl of each transformed cell culture were plated and spread with glass beads on LB 1.5% agar medium with gentamicin added at 20µg/ml and incubated at 37ºC overnight. Once the colonies grew well, it was necessary to do a

screening of some colonies through “patching”, due to the fact that the ligation was made using both pDE00.0113 backbone and the pro-HNop inserts. The patching consists in taking with a tip as many colonies as possible (10-50) from the last culture, then drawing a small “X” with each colony, making sure each colony is well separated from each other, on the same medium used before and also on another medium with kanamycin added at 50µg/ml instead gentamicin (because of pDE00.0113 backbone has a kanamycin resistance gene (Figure 5.3a)), being careful that all colonies are in the same position in both mediums (assigning numbers to each colony might be useful). Then the plates were incubated at 37ºC overnight. After that, colonies that grew well on both mediums probably are “false positives”, since pDE00.0113 backbone was ligated to pDU97.1005 binary vector, conferring to the colony resistance to both antibiotics. On the other hand, colonies that grew only on the medium with gentamicin were selected as “positive colonies”.

However, to make sure about that, colony PCR (Annex A, Protocol 4) was used to test each possible positive colony, with the corresponding primers to each HNop construct and CaMV35S-2 primer (Table 5.1). Once positive colonies were identified by colony PCR, they were grown in LB medium with gentamicin added at 20µg/ml and incubated at 37ºC overnight with shaking. Subsequently, the plasmid DNA of each cell culture was obtain as previously described using QIAPREP® Spin MINIprep Kit (cat# 27104) and measured with the NanoDrop 1000®. In order to make sure all the colonies have the pDU97.1005 binary vector with each pro-HNop insert, a digestion (Annex A, Protocol 1) with AscI was made to each plasmid DNA, then 3µl of each digested plasmid DNA with 2µl of loading buffer were loaded in a 0.8% agarose gel and visualized it as described in annex A, protocol 2. The presence of a ~4kb band was considered as positive colony, since is the size of each pro-HNop insert. Finally, the cell culture of one positive colony with its corresponding p35S:HNop binary vector (p35S:HNop1, p35S:HNop2, p35S:HNop3 or p35S:HNop4; where “p35S:HNop” means the pDU97.1005 binary vector ligated with any pro-HNop insert) was used to make freezer stocks as described above. The plasmid DNA obtained of each p35S:HNop binary vector, was used to transform *Agrobacterium tumefaciens* competent cells (EHA105:pCH32 strain), kindly supplied by Dr. Dandekar’s lab, as shown in annex A protocol 6 and they were used to perform the agroinfiltration.

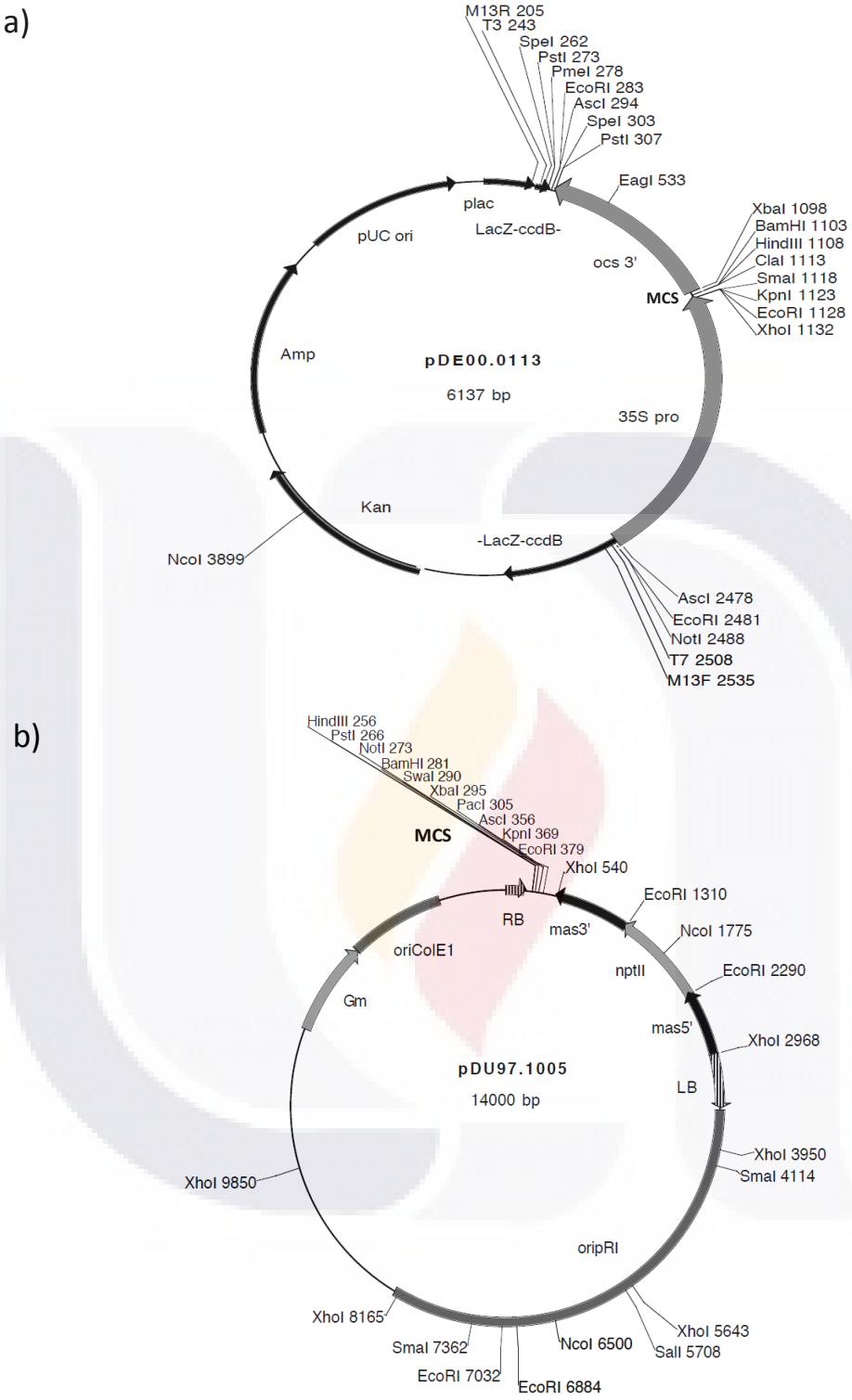


Figure 5.3 Plasmid maps used to make the 35S binary vector. a) pDE00.0113 vector, which was made using the expression cartridge from pART7 (Gleave, 1992) that comprises the cauliflower mosaic virus Cabb B-JI isolate 35S promoter (35Spro), a multiple cloning site (MCS) and the transcriptional termination region of the octopine synthase gene (ocs3'). The cartridge is located between the *lacZ'* region and is represented with grey arrowed boxes that denote orientation of coding region. The Ampicilyn (Amp) and Kanamycin (Kan) bacterial selectable marker and the replication origin pUC are represented by dark arrows, which show their orientation. b) Binary vector, pDU97.1005. The right border (RB) and left border (LB) are indicated by the

lines across arrowed boxes. The chimeric *nptII* region is shaded (arrows denote orientation of coding region). The oripRI replicon for maintenance in *Agrobacterium*, the ColE1 origin of replication for high-copy maintenance in *Escherichia coli,* and the MCS are indicated. Gentamycin (Gm) bacterial selectable marker is indicated by a grey arrowed box that denotes its orientation.

Table 5.1 Sequence of the primers used to perform the colony PCR. The HNop's primers where design using the NCBI-primer software and were synthesized by SigmaAldrich. The primers CaMV35S-2 and TRBO-1 were kindly supplied by Dr. Dandekar's lab, while pMCSdelQAN-1 primer was kindly supplied by Dr. Falk's lab. Note: HNop2 primer was used to amplify either HNop2 or HNop4 sequence.

Primer	Orientation	Sequence (5'>3')
HNop1	Reverse	Cggggaaccaaacacggtca
HNop2	Reverse	Tcgtgatactgcccatcaaagcc
HNop3	Reverse	atacactgggaaccaaacacggtc
CaMV35S-2	Forward	Gacgtaagggatgacgcacaat
TRBO-1	Forward	Ctactgtcgccgaatcggattcg
pMCSdelQAN-1	Forward	Catggatgcttctccgcgag

5.3.3 Cloning of HNop1-4 constructs into pTRBO binary vector

In order to generate binary vectors with each HNop construct into a TMV-based vector (pTRBO:HNop1, pTRBO:HNop2, pTRBO:HNop3 and pTRBO:HNop4), freezer stocks of pUC57:HNop1-4 vectors and pJL TRBO binary vector (Figure 5.4; kindly supplied by Dr. Dandekar's lab), were grown in 5ml of LB medium with ampicillin added at 100µg/ml (pUC57:HNop1-4) and in 2x 10ml of LB medium with kanamycin added at 50µg/ml for pJL TRBO (useing50ml Falcon tubes) and placed at 37ºC overnight with 225rpm of shaking. Then, 2ml of each cell culture of pUC57:HNop was placed into a 2ml centrifuge tube and spun it down for 1min at 13,000 rpm, and for pJL TRBO both 10ml cell culture tubes were centrifuged for 10 min at 9,000rpm. After that, the supernatant was decanted and the pellet was processed to obtain plasmid DNA using QIAPREP® Spin MINIprep Kit (cat# 27104). Once the plasmid DNA for each cell culture was obtained, the concentration was measured using a NanoDrop 1000®. First to free each HNop construct from the pUC57 vector, the plasmid DNA of each one was cut with PacI and AvrII restriction enzymes, which corresponds to MCS located in pJL TRBO (Figure 5.4), which was also digested with the same enzymes (Annex A, Protocol 1).

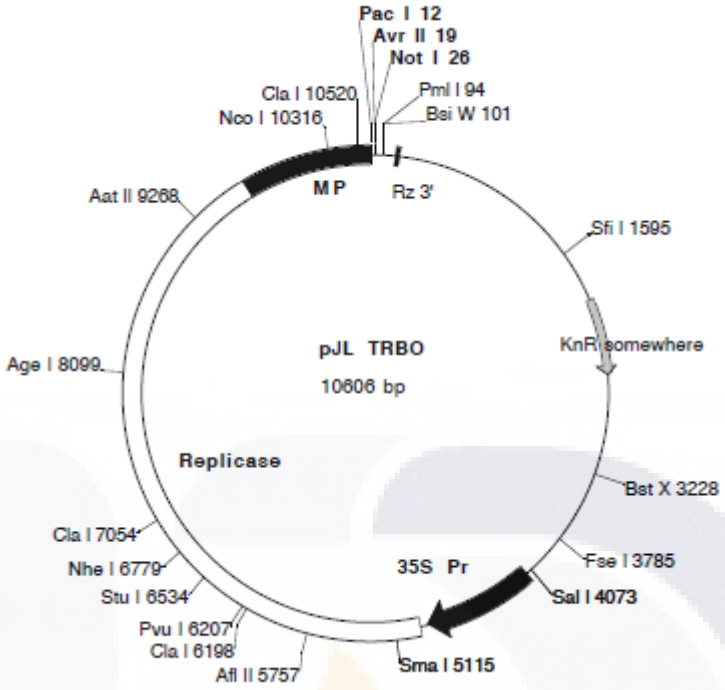


Figure 5.4 Plasmid map of pJL TRBO binary vector (Lindbo 2007). pJL TRBO (**T**obacco Mosaic Virus **R**NA **B**ased **O**verexpression) is based on the mini binary vector pCB301 (Xiang et al 1999), which has T-DNA borders (not shown), but no vir genes, or genes needed for conjugation into other bacteria. Capcide protein sequence from TMV RNA was replaced with a multiple cloning site (PacI, AvrII, and NotI). Replicase, TMV 126K/183K ORF, is shown as a white box. The movement protein (MP), ribozyme (Rz) and CaMV 3' polyA signal/transcription terminator (3') are indicated by a black box. The duplicated CaMV 35S promoter (35S Pr) is represented by a black arrowed box that denotes the orientation of coding region, and kanamycin resistence gene (KnR) is located somewhere in the pCB301 backbone.

Once the plasmid DNA of each HNop construct and the vector were cut with the appropriate restriction enzymes (PacI and AvrII), 20µl of each digestion reaction was loaded (except for pJL TRBO vector's reaction 5µl were loaded) with 2µl of loading buffer in a 0.8% agarose gel and the electrophoresis was performed as in annex A, protocol 2 (Figure 5.5). When the four HNop constructs were able to be isolated from the pUC57 vector, the respective bands (~2kb) were excised from the gel using a sharp scalpel and placed in separate 1.5ml centrifuge tubes. Then, the DNA was recovered from each gel slice using the QIAquick Gel Extraction Kit (cat# 28704). The pJL TRBO binary vector was not recovered, since it was just run to make sure it had been digested correctly by the restriction enzymes. After that and in order to insert each HNop construct in the pJL TRBO binary vector, a ligation was performed following annex A, protocol 3. Once the ligation was made, One Shot® DH5α-TI^{R} Chemically Competent *E. coli* cells were transformed, following the company's protocol, with each

HNop construct previously ligated into the pJL TRBO binary vector. Then, to select the transformed cells with the four different binary vectors generated (pTRBO:HNop1, pTRBO:HNop2, pTRBO:HNop3, and pTRBO:HNop4), 10µl of each cell culture were plated and spread with glass beads on LB 1.5% agar medium added with kanamycin 50µg/ml as selective agent and incubated at 37ºC overnight.

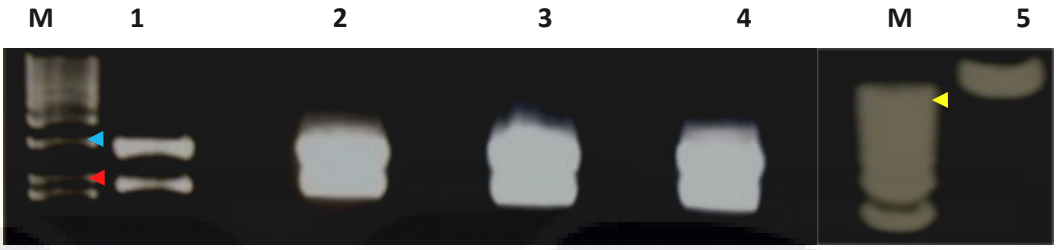


Figure 5.5 Plasmid DNA of pUC57:HNop1-4 and pJL TRBO digested with PacI and AvrII restriction enzymes. A 0.8% agarose gel was used to visualize and recover bands of HNop1-4 constructs (~2kb). M) 1 kb Plus DNA Ladder (Invitrogen cat#10787-018), 1) HNop1 (1.779kb), 2) HNop2 (1.860kb), 3) HNop3 (1.791 kb), 4) HNop4 (1.837kb), and 5) pJL TRBO binary vector (10.606kb). Red, blue and yellow arrows stand for 2kb, 3kb and 10kb respectively.

After that, positive colonies were selected through colony PCR (Annex A, Protocol 4). Appropriate primers (Table 5.1) were used to amplify from 150nt upstream of pJL TRBO's MCS, until a little more than the middle of each HNop sequence, to obtain a PCR product of ~1kb, which indicates that the HNop construct was successfully ligated within the pJL TRBO binary vector and is present into the colony. Once positive colonies were identified by colony PCR, they were grown in LB medium with kanamycin added at 50µg/ml and incubated at 37ºC overnight with 225rpm of shaking. Subsequently, the plasmid DNA of each cell culture was obtained as previously described using QIAPREP® Spin MINIprep Kit (cat# 27104) and measured with the NanoDrop 1000®. In order to make sure all the colonies selected as positive have the pJL TRBO binary vector with each HNop construct, a digestion (Annex A, Protocol 1) with PacI and AvrII was made to each plasmid DNA, then 3µl of each digested plasmid DNA with 2µl of loading buffer were loaded in a 0.8% agarose gel and visualized it as described in annex A, protocol 2. The presence of a ~2kb band was considered as positive colony, since it is the size of each HNop construct. Subsequently, the cell culture of one positive colony with one of pTRBO:HNop binary vectors was used to make freezer stocks as described earlier. Finally, plasmid DNA obtained of each pTRBO:HNop binary vector was used to transform *Agrobacterium tumefaciens* competent cells (EHA105:pCH32 strain), kindly supplied by Dandekar's lab, as shown in annex A protocol 6 and these recombinant agrobacteria were used to perform the agroinfiltration.

5.3.4 Cloning of HNop1-4 constructs into pCMVva binary vector

In order to generate binary vectors with each HNop construct into a Cucumber Mosaic Virus (CMV)-based vector (pCMVva:HNop1, pCMVva:HNop2, pCMVva:HNop3 and pCMVva:HNop4), freezer stocks of pUC57:HNop1-4 vectors and pMCSdelQAN binary vector (kindly supplied by Dr. Falk's lab, Department of Plant Phatology, UCDavis), that corresponds to the RNA-3 of CMV's tripartite genome (Figure 5.6a), were cultured in 5ml of LB medium with ampicillin added at 100µg/ml (pUC57:HNop1-4) and in 10ml of LB medium with kanamycin added at 50µg/ml for pMCSdelQAN (using 50ml Falcon tubes) and placed at 37ºC overnight with 225rpm of shaking. Then, 2ml of each cell culture of pUC57:HNop was placed into a 2ml centrifuge tube and spun it down for 1min at 13,000 rpm, and for pMCSdelQAN the 10ml cell culture tube was centrifuged for 10 min at 9,000rpm. After that, the supernatant was decanted and the pellet was processed to obtain plasmid DNA using QIAPREP® Spin MINIprep Kit (cat# 27104). Once the plasmid DNA for each cell culture was obtained, the concentration was measure using a NanoDrop 1000®. First to free each HNop construct from the pUC57 vector, the plasmid DNA of each one was digested with PstI and HindIII restriction enzymes, which corresponds to MCS located in pMCSdelQAN (Figure 5.6a), which was also digested with the same enzymes (Annex A, Protocol 1).

Once the plasmid DNA of each HNop construct and the vector were digested with the appropriate restriction enzymes (PstI and HindIII), 20µl of each digestion reaction was loaded (except for pMCSdelQAN's reaction 5µl were loaded) with 2µl of loading buffer in a 0.8% agarose gel and the electrophoresis was performed as in annex A, protocol 2 (Figure 5.7). When the four HNop constructs were able to be isolated from the pUC57 vector, the respective bands (~2kb) were excised from the gel using a sharp scalpel and placed n separate 1.5ml centrifuge tubes. Then, the DNA was recovered from each gel slice using the QIAquick Gel Extraction Kit (cat# 28704). The pMCSdelQAN binary vector was not recovered, since it was just run to make sure it had been digested correctly by the restriction enzymes. After that and in order to insert each HNop construct in the pMCSdelQAN binary vector, a ligation was performed following annex A, protocol 3. Once the ligation was made, One Shot® DH5α-TIR Chemically Competent E. coli cells were transformed, following the company's protocol, with each HNop construct previously ligated into the pMCSdelQAN binary vector. Then, to select the transformed cells with the four different binary vectors generated (pCMVva:HNop1, pCMVva:HNop2, pCMVva:HNop3, and pCMVva:HNop4), 10µl of each cell culture were plated and spread with glass beads on LB 1.5% agar medium with kanamycin added at

50µg/ml and incubated at 37ºC overnight.

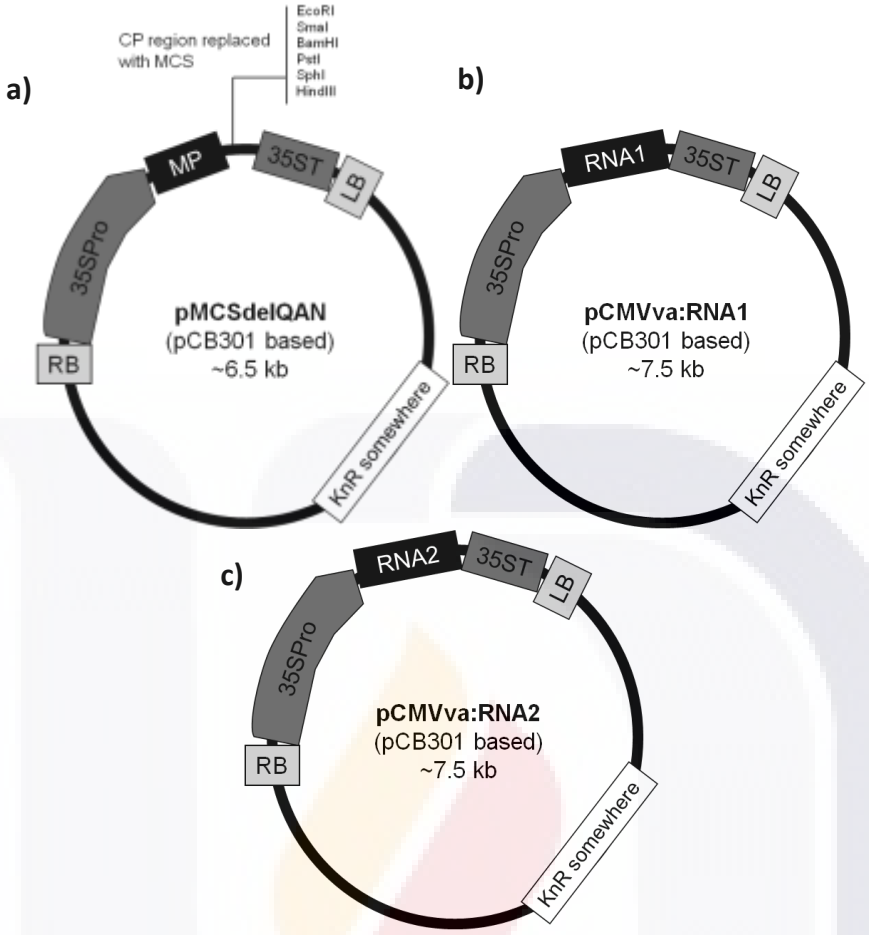


Figure 5.6 Plasmid map of Cucumber Mosaic Virus viral amplicon (CMVva) system. The mini binary vector pCB301 (Xiang et al 1999) was engineered to carry CMV tripartite genome (RNA1, RNA2 and RNA3) separately. **a)** CMV RNA3 was used to generate pMCSdelQAN binary vector, moreover capcide protein (CP) sequence was replaced with a multiple cloning site (MCS). The movement protein (MP) is indicated by a black box; **b)** CMV RNA1 (indicated by a black box) was used to generate pCMVva:RNA1 binary vector; **c)** CMV RNA2 (indicated by a black box) was used to generate pCMVva:RNA2 binary vector. The right border (RB) and left border (LB) are indicated by light grey boxes. The CaMV 35S promoter (35SPRO) is indicated by a grey arrowed box that denotes the orientation of the coding region, and CaMV 3' polyA signal/transcription terminator (35ST) is shown by a grey box. The kanamycin resistence gene (KnR) is located somewhere in the pCB301 backbone.

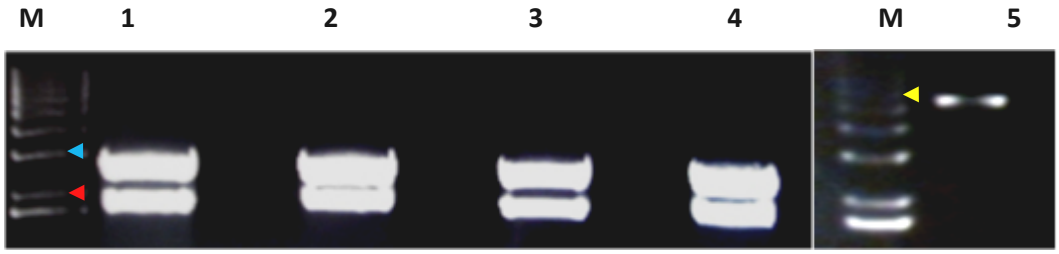


Figure 5.7 Plasmid DNA of pUC57:HNop1-4 and pMCSdelQAN digested with PstI and HindIII restriction enzymes. A 0.8% agarose gel was used to visualize and recover bands of HNop1-4 constructs (~2kb). M) 1 kb Plus DNA Ladder (Invitrogen cat#10787-

018); 1) HNop1 (1.779kb); 2) HNop2 (1.860kb); 3) HNop3 (1.791 kb); 4) HNop4 (1.837kb); and 5) pMCSdelQAN binary vector (6.5kb). Red, blue and yellow arrows stand for 2kb, 3kb and 6kb respectively.

After that, positive colonies were selected through colony PCR (Annex A, Protocol 4). Appropriate primers (Table 5.1) were used to amplify from 60nt upstream of pMCSdelQAN's MCS, until a little more than the middle of each HNop sequence, to obtain a PCR product of ~1kb, which indicates that the HNop construct was successfully ligated within the pMCSdelQAN binary vector and is present into the colony. Once positive colonies were identified by colony PCR, they were grown in LB medium with kanamycin added at 50µg/ml and incubated at 37ºC overnight with 225rpm of shaking. Subsequently, the plasmid DNA of each cell culture was obtained as previously described using QIAPREP® Spin MINIprep Kit (cat# 27104) and measured with the NanoDrop 1000®. In order to make sure all the colonies selected as positive have the pMCSdelQAN binary vector with each HNop construct, a digestion (Annex A, Protocol 1) with PstI and HindIII was made to each plasmid DNA, then 3µl of each digested plasmid DNA with 2µl of loading buffer were loaded in a 0.8% agarose gel and visualized it as described in annex A, protocol 2. The presence of a ~2kb band was considered as positive colony, since it is the size of each HNop construct. Finally, the cell culture of one positive colony of each of the four pCMVva:HNop binary vectors was used to make freezer stocks as described earlier. Plasmid DNA obtained of each pCMVva:HNop binary vector was used to transform *Agrobacterium tumefaciens* competent cells (GV3101 strain), kindly supplied by Dr. Falk's lab, as shown in annex A protocol 6 and these agrobacteria were used to perform the agroinfiltration.

5.4 VACUUM AGROINFILTRATION OF HARVESTED *NICOTIANA BENTHAMIANA* LEAVES.

5.4.1 Growth of recombinant *Agrobacterium*, agroinfiltration and incubation conditions

As seed culture, freezer stock generated before of *Agrobacterium,* transformed with the different binary vectors, were cultured first in 5ml of LB medium with appropriate antibiotics added (Table 5.2) at 28ºC for 20-24h with 250rpm of shaking and then scaled up to 100ml of LB medium with the same growth conditions. The *p19* gene silencing suppressor from *Tomato Bushy Stunt Virus* (Voinnet et al. 2003) cDNA into *A. tumefaciens* C58C1 strain was co-infiltrated with the recombinant *Agrobacterium* generated in this study (Table 5.2) to improve the expression level of the HN protein.

Vacuum infiltration equipment consisted of a cylinder-like plastic chamber (diameter 29.2cm, height 33cm) and a vacuum valve that was connected to an in-house vacuum system. Prior to agroinfiltration, 5-6 weeks old *N. benthamiana* plants were brought from the green house and the leaves were detached directly before to be use. The experiments (agroinfiltration) were accomplished with each expression system harboring each HNop construct twice and individually. Three detached leaves were used per condition to evaluate. The agroinfiltration process was performed as described in figure 5.8. Because of CMV has a tripartite genome (RNA1, RNA2 and RNA3), which were engineered and cloned in separately plasmids (Figure 5.6), agroinfiltration was carried out mixing each cell culture (OD_{600}=1.0) of CMV RNAs and *p19* (1:1:1:1) to get a final OD_{600}=0.25 for each.

For incubation, plastic containers were adapted as humidity chambers to keep agroinfiltrated leaves healthy. To begin, ~400g of Perlite (E.B. Stone) were placed in a plastic pitcher and filled with water, and then it was stirred and let it soak for 1-2h. After that, the excess of water was poured off, and then the Perlite was used to form a ~3cm deep layer inside the plastic container, which was previously wrapped with aluminum foil to protect from light. Finally, a metallic mesh (4sq/inch^2) was used to create a platform suspended ~2cm above Perlite. To do that, ends of the mesh were bended and buried in Perlite for support.

Table 5.2 Growth conditions for all binary vectors generated and transformed into *Agrobacterium tumefaciens*. Antibiotics: Rf= Rifampicin; Tn= Tetracycline; Gm= Gentamicin; Kn= Kanamycin.

Binary vector	Agrobacterium strain	Antibiotic (µg/ml)
p35S:HNop1	EHA105:pCH32	Rf10+Tn10+Gm20
p35S:HNop2	EHA105:pCH32	Rf10+Tn10+Gm20
p35S:HNop3	EHA105:pCH32	Rf10+Tn10+Gm20
p35S:HNop4	EHA105:pCH32	Rf10+Tn10+Gm20
pTRBO:HNop1	EHA105:pCH32	Rf10+Tn10+Kn50
pTRBO:HNop2	EHA105:pCH32	Rf10+Tn10+Kn50
pTRBO:HNop3	EHA105:pCH32	Rf10+Tn10+Kn50
pTRBO:HNop4	EHA105:pCH32	Rf10+Tn10+Kn50
pCMVva:HNop1	GV3101	Rf10+Gm20+Km50
pCMVva:HNop2	GV3101	Rf10+Gm20+Km50
pCMVva:HNop3	GV3101	Rf10+Gm20+Km50
pCMVva:HNop4	GV3101	Rf10+Gm20+Km50
pCMVva:RNA1	GV3101	Rf10+Gm20+Km50
pCMVva:RNA2	GV3101	Rf10+Gm20+Km50
p19	C58C1	Tn10+Kn50

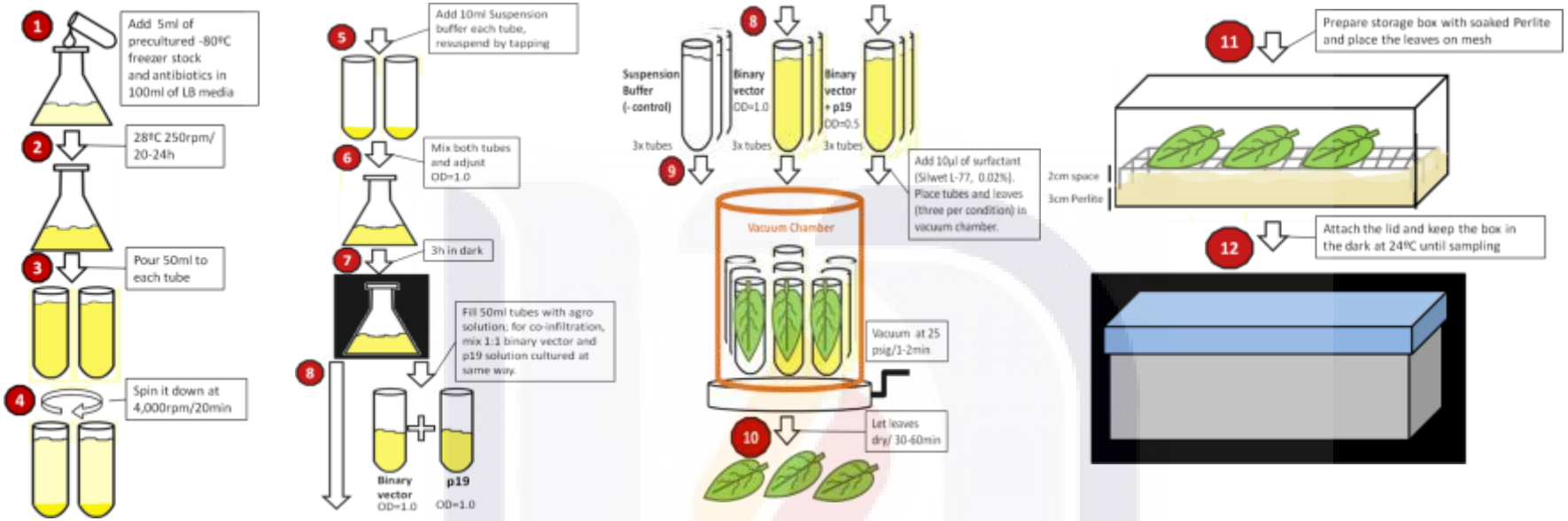


Figure 5.8 Scheme of the biomanufacturing platform SwiftVax® (vacuum agroinfiltration), in 5-6 weeks old harvested *N. benthamiana* leaves. All the experiments were carried out with three leaves for each binary vector generated previously in this study (Table 5.2). **1)** and **2)** To begin, ~200µl of *Agrobacterium* -80ºC freezer stock carrying the binary vector (p35S, pTRBO or pCMVva), with each of the HNop constructs (HNop1, HNop2, HNop3 or HNop4) and *p19* gene silencing suppressor were precultured in 5ml of LB medium added with antibiotics (depends of the binary vector and *Agro* strain, see table 5.2), at 28ºC for 20-22h with 250rpm of shaking. After that, precultured 5ml of each cell culture were placed in 100ml of LB medium added with same antibiotics and cultured same way. **3)** After 20-22h of incubation, the 100ml of each cell culture were divided equally in two 50ml tubes and labeled. **4)** All tubes were centrifuge at 4,000rpm for 20min at room temperature. Once pellet was clearly seen, it was kept in the bottom and supernatant was decanted. **5)** In order to resuspend the pellet by tapping and to measure bacteria concentration, 10ml of sterile suspension buffer (10mM MES pH 5.6, 10mM MgCl2, and 150µM acetosyringone) were added to each tube. **6)** Then, each resuspended pellet was mix with its similar into a new sterile flask. Subsequently, the optic density (OD_{600}) was measured and adjusted to 1.0 adding the required suspension buffer. **7)** Each flask with the different binary vectors and with the *p19* were incubated in the dark at room temperature for 3h. **8)** Prior performing of vacuum agroinfiltration, each *Agrobacterium* cell suspension (OD_{600}=1.0) was either mix with an equally volume (1:1) of *p19* (OD_{600}=1.0) to get a final OD_{600}=0.5, or they were used alone with a final OD_{600}=1.0. In the case of pCMVva system, plasmids with CMV's RNA1 and RNA2 were cultured same way, mix (1:1:1) with pCMVva:HNop binary vector and use alone (OD600=0.33) or mix (1:1:1:1) with p19 for co-infiltration (OD600=0.25). As negative control, suspension buffer was employed. 9) The surfactant Silwet L-77 was added at a final concentration of 0.02% (10µl per tube), to reduce the surface tension of the solution and facilitate the agroinfiltration. Then, leaves were submerged into the bacterial suspension and subjected to a vacuum, which was set at 27" Hg for 1 - 2min and then quickly released . 10) After the vacuum was released, the plant material was removed from the suspension and allowed to dry inside of the biosafety cabinet for 30-60min. 11) Once the agroinfiltrated leaves were completely dried, they were placed on the mesh of humidity chamber prepared prior agroinfiltration (see section 5.4.1) and the lid of the container was attached securely. 12) The sealed container was cover with a plastic bag and kept it in a controlled environmental chamber at 20±2ºC for up to 6 days; sampling was made in 2, 4 and 6 day post infiltration (DPI).

5.5 PROTEIN EXPRESSION KINETIC OF AGROINFILTRATED *NICOTIANA BENTHAMIANA* LEAVES

5.5.1 Sampling and HN protein extraction

Leaf discs were sampled from agroinfiltrated leaves after 2, 4, and 6 days post-infiltration (DPI), using the lid of a 1.5ml centrifuge tube per leaf. Each sample was comprised of two-three discs (~20mg total) obtained from the same leaf (Figure 6.7). Phosphate buffer saline (PBS) composed of 137 mM NaCl, 2.7 mM KCl, 10.1 mM Na_2HPO_4 and 1.8 mM KH_2PO_4 (pH 7.4) was used for extraction. Pre-cooled PBS was added to the leaf discs at a ratio of 10 µL per mg of fresh weight leaf tissue. Cells were lysed on ice using a sterile plastic pestle attached to a drill (Makita). During the lysing tubes were kept on ice. Then the cell lysate was clarified by centrifugation at 4ºC and 14,000rpm for 20 min. and the supernatant was collected and immediately assayed for total soluble protein with the Bradford Assay. In order to increase the amount of recollected HN protein, 100µl of cell lysate prior clarification were sonicated for 20min at 4ºC (Sonicor), and then they were clarified and analyzed through Bradford assay like said previously. Supernatants were stored at −80ºC until required for ELISA and Western Blot analysis.

5.5.2 Bradford analysis of total soluble protein (TSP).

The amount of TSP present in the plant extracts was quantified immediately after protein extraction, using the Bradford Protein Assay (Bradford 1976) and following Bio-Rad Protein Assay protocol (Annex A, Protocol 7). Samples and controls diluted (dilution factor= 5) in phosphate buffer saline (PBS) were applied directly to the microplate wells (Costar 3598). For each microplate, a standard curve was generated with 0.5, 0.4, 0.3, 0.2, 0.1, 0.05, and 0 mg/mL of bovine serum albumin diluted in PBS. After adding Bradford Protein Reagent (BioRad, Hercules, CA), the microplates were incubated at room temperature for 10 min, and absorbance was measured at 595 nm. Each sample and control was assayed in triplicate, and total soluble protein concentrations were interpolated from the standard curve.

5.6 HN PROTEIN EXPRESSION LEVEL IN AGROINFILTRATED *N. BENTHAMIANA* LEAVES.

5.6.1 ELISA analysis.

Direct Enzyme-Linked Immunosorbent assay (ELISA) was used to quantify the amount of plant-expressed HN protein. To realize it, microplate wells (Corning Costar 3590, Corning, NY) were coated with samples (diluted 1:30 for TRBO and 35S and 1:20 for CMVva) and controls (diluted 1:5) in phosphate buffer saline (PBS) pH 7.4 as well as for each microplate, a standard curve was generated with 5000; 1666.7; 555.6; 185.2; 61.7; 20.6; 6.9; and 2.3 ng/ml of C-terminal FLAG–BAP™ fusion protein (Sigma-Aldrich cat#P7457) diluted in PBS. Wells were loaded with 50µl per well of samples, controls and standards (each sample and control was analyzed in triplicate) and then incubated for 1 h at 37ºC. The plate was rinsed with PBS with 0.05% TWEEN 20 four times, to eliminate excess of samples and controls. Blocking was achieved by a 15min incubation with 5% nonfat dry milk (Safeway) diluted in PBS. The plate was again rinsed with PBS with 0.05% TWEEN 20 four times, to eliminate excess of blocking solution. Afther that, mouse mAb ANTI-FLAG® M2 conjugated with horseradish peroxidase (HRP) (Sigma Aldrich cat#A8592) diluted 1:2000 in PBS was added, and the microplates were incubated at 37 °C for 1 h.

Detection was performed with TMB (3, 3´, 5, 5´ tetramethylbenzidene) liquid substrate for ELISA. After 10min the reaction was stopped with 50µl per well of 1N HCl and the absorbance was immediately measured at 450 nm. HN concentrations were interpolated from the linear portion of the standard curve. For further information see annex A, protocol 8.

5.7 CHARACTERIZATION OF HN PROTEIN.

5.7.1 Western blot assay.

Protein samples and controls were denatured by heating for five min at 95ºC in 5X sodium dodecyl sulfate-polyacrilamide gel (SDS-PAGE) sample buffer C and 1M dithiothreitol (DTT), for reduction of proteins at a final concentration of 90 mM DTT, and the standard C-terminal FLAG–BAP™ fusion protein (Sigma-Aldrich cat#P7457) was processed following the company's recommendations. Thirty microliters of sample, control and standard were loaded in a 4-15% mini-protean TGX™ gel (Bio Rad cat# 456-1083S) and fractionated with SDS-PAGE at 200 V for 30 min, and transferred to a

0.45 µm nitrocellulose membrane (Bio Rad cat# 162-0091) at 120 V for 80 min. Blots were blocked with 5% non-fat dry milk (Safeway) diluted in 1X PBS for 15min at room temperature. After that, membrane was washed with 1X PBST (1X PBS with 0.05% TWEEN 20) three times, to eliminate excess of blocking solution. Immunoblotting was performed using mouse mAb ANTI-FLAG® M2 conjugated with horseradish peroxidase (HRP) (Sigma Aldrich cat#A8592) diluted 1:2000 in 5% non-fat dry milk (Safeway) diluted in 1X PBS. The blot was developed using TMB stabilized substrate (Promega cat#W4121) for horseradish peroxidase on membranes. For further information see annex A, protocol 9.

5.8 STATISTICAL ANALYSIS

Statistical analyses were conducted using STATGRAPHICS Centurion XV 15.2.05 for the calculation of p-value of t-test. Uncertainties associated with calculated mean values were reported as the standard deviation. Graphics were performed using Microsoft Excel 2007. All statistical analyses are shown more detailed in annex C.

6. RESULTS

6.1 SELECTION OF NDV STRAIN AND ENGINEERING OF HN cDNA TO BE TRANSIENTLY EXPRESSED.

6.1.1 Multiple sequence alignment for HN protein of different NDV strains.

In order to determine what strain will be used to engineer the HN cDNA, we based our selection on a previous work by Miller and coworkers (2007), where they showed the CA02 strain's potential as wide-range vaccine against different NDV genotypes. To find differences in the epitope region between strains that belong to different genotypes, a multiple sequence alignment was performed. Once done, it was able to identify that there are punctual mutations in two of the three epitopes for strains that belong to genotype V: gamefowl/ US (CA)/ 212676/ 02 (CA02) strain in I352V and S520T, chicken/ Mexico/ 37821/ 96 (MX96) strain in I352V and S520I, and P05 strain in E347G and I352V (Figure 6.1). Meanwhile, either strains that belong to genotype II (APMV-1/chicken/U.S.(TX)/GB/1948 (Texas GB); BC-45; LaSota; Takaaki/B1; VG/GA) or to genotype IX (JS/1/97/China) do not have mutation within the epitopes, even if some of them are classified as velogenic strains (Figure 6.1).

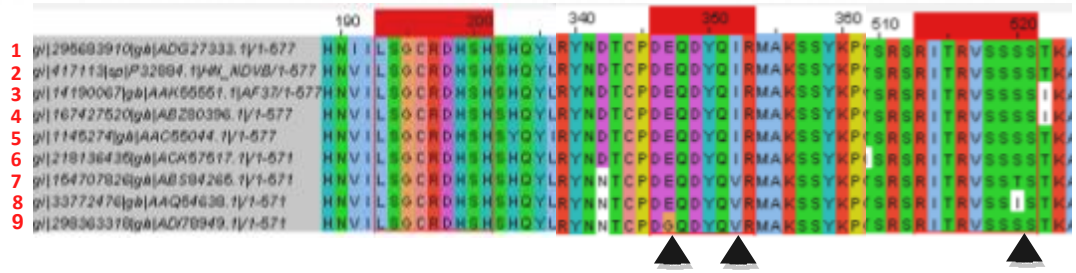


Figure 6.1 Multiple sequence alignment of NDV-HN protein sequence of different strains, which belong to different genotypes and some of them are currently used as commercial vaccines (2-5 and 9 strains). The ClustalW2 software was used to identify punctual mutation within the three epitope, which are underlined (193-201, 346-353, and 513-521). **1** Texas GB strain (Access # ADG27333) velogenic genotype II; **2** BC-45 strain (Access # P32884) velogenic genotype II; **3** Takaaki-B1 strain (Access # AAK55551) lentogenic genotype II; **4** VG/GA strain (Access # ABZ80396) genotype II; **5** LaSota strain (Access # AAC55044) lentogenic genotype II; **6** JS/1/97/China strain (Access # ACK57517) genotype IX; **7** CA02 strain (Access # ABS84265) velogenic genotype V; **8** MX96 strain (Access # AAQ54638) velogenic genotype V; **9** P05 strain (Access # ADI78949) genotype V. Black arrows indicate punctual mutations within epitope regions for 7, 8 and 9 strains.

6.1.2 Engineering and codon optimization of HN cDNA.

Codon optimization was performed for the HN cDNA sequence of CA02 strain to improve its expression level in *N. benthamiana* leaves. To achieve this, the Visual Gene Developer software (Jung and McDonald 2011) was used, which carried out the optimization using a *N. benthamiana* codon-usage table and for the analyses of optimized HN (HNop) constructs the codon adaptation index (CAI), Effective number of codons (Nc), and the guanine and cytosine content (GC %) were considered (Table 6.1). Nc was kept very similar from the original, only increasing in HNop1 from 56.09 to 58.17 and decreasing around 1.5 in the rest (Table 6.1). CAI was not too much adjusted from the original 0.68 to 0.72-0.75 in all HNop constructs as well as GC content kept like from original ~45.5 to 44.5 % (Table 6.1). Other features such as number of mismatched bases and codons, repeated DNA sequences, potential polyadenylation signals, , and unwanted restriction enzyme sites were compared between original and optimized HN constructs and can be seen more detalid in Annex B analysis 1.

In addition, different sequences known to enhance the expression and accumulation level of recombinant proteins in plant tissue, were inserted and optimized to obtain four different HN construct such as the psaDb 5'UTR, three different signal peptides, 3xFLAG-tag sequence, and KDEL sequence. Also restriction sites were added to the 5' and 3' ends. And as result four different optimized HN (HNop) constructs were generated (Figure 6.2). The size of each construct varied by a small number of base

pairs: HNop1= 1,779bp; HNop2= 1,860bp; HNop3= 1,779bp and HNop4=1,837bp. The sequence of each HNop construct and the native HN gene are shown in Annex B, table 1.

Figure 6.2 Schematic diagram of optimized HN (HNop) constructs synthesized. All the constructs were engineered and codon optimized to improve their expression levels in vacuum agroinfiltrated *Nicothiana benthamiana* leaves. psaDb) the untranslated 5' sequence (5'UTR) of *psaDb* gene from *N. sylvestris* (Yamamoto et al 1995), not present in HNop4; SP-HN) Signal peptide of HN protein's first 48 N-term aminoacids; 2S2) Signal peptide of storage albumin from *Arabidopsis thaliana*; RAmy) Signal peptide of rice alpha-amylase (Huang et al 2004).; KDEL) endoplasmic reticulum retention sequence (not present in HNop3); 3xFLAG) fusion protein sequence placed in all the constructs before of HN sequence's 49 N-term amino acid and after of the signal peptide. Also added was a multiple cloning site in 5' (PacI-XhoI-PstI) and 3' (HindIII-SpeI-AvrII).

Table 6.1 Codon optimization of HN constructs using Visual Gene Developer software (Jung and McDonald 2011). Codon optimization was performed using *N. benthamiana* codon-usage table only in open reading frames (ORFs). Three main features were considered during codon optimization: GC content (%), Codon Adaptation Index (CAI), and Effective number of codons (Nc).

	HN Constructs before optimization				Optimized HN (HNop) constructs			
	1	2	3	4	1	2	3	4
GC content %	**45.78**	**45.39**	**45.93**	**45.47**	**44.44**	**44.44**	**44.59**	**44.44**
CAI	**0.68**	**0.68**	**0.69**	**0.68**	**0.72**	**0.75**	**0.74**	**0.75**
Nc	**56.09**	**56.59**	**56.77**	**56.65**	**58.17**	**55.42**	**55.11**	**55.42**

6.1.3 Cloning of HNop constructs into p35S, pTRBO and pCMVva binary vector.

In order to express the HNop constructs into harvested leaves, they were first released from the pUC50 vector using appropriate restriction enzymes for the MCS (Figure 5.2,

5.7 and 5.9). Subsequently, they were ligated into different binary vectors (p35S, pTRBO and pCMVva) and then transformed into *Escherichia coli*. After that, molecular tests such as DNA digestion and colony PCR were performd to select a single colony that was carrying the different binary vectors with the HNop constructs (Figure 6.3). Once positive colonies were selected, the plasmid DNA was used to transform different *Agrobacterium tumefaciens* strains (Table 5.2). Finally, freezer stocks of *Agrobacterium,* harboring the different binary vectors with the four HNop constructs, were made to be used during agroinfiltration. Maps of the T-DNA regions of various modified binary vectors used in this study are shown in Figure 6.4.

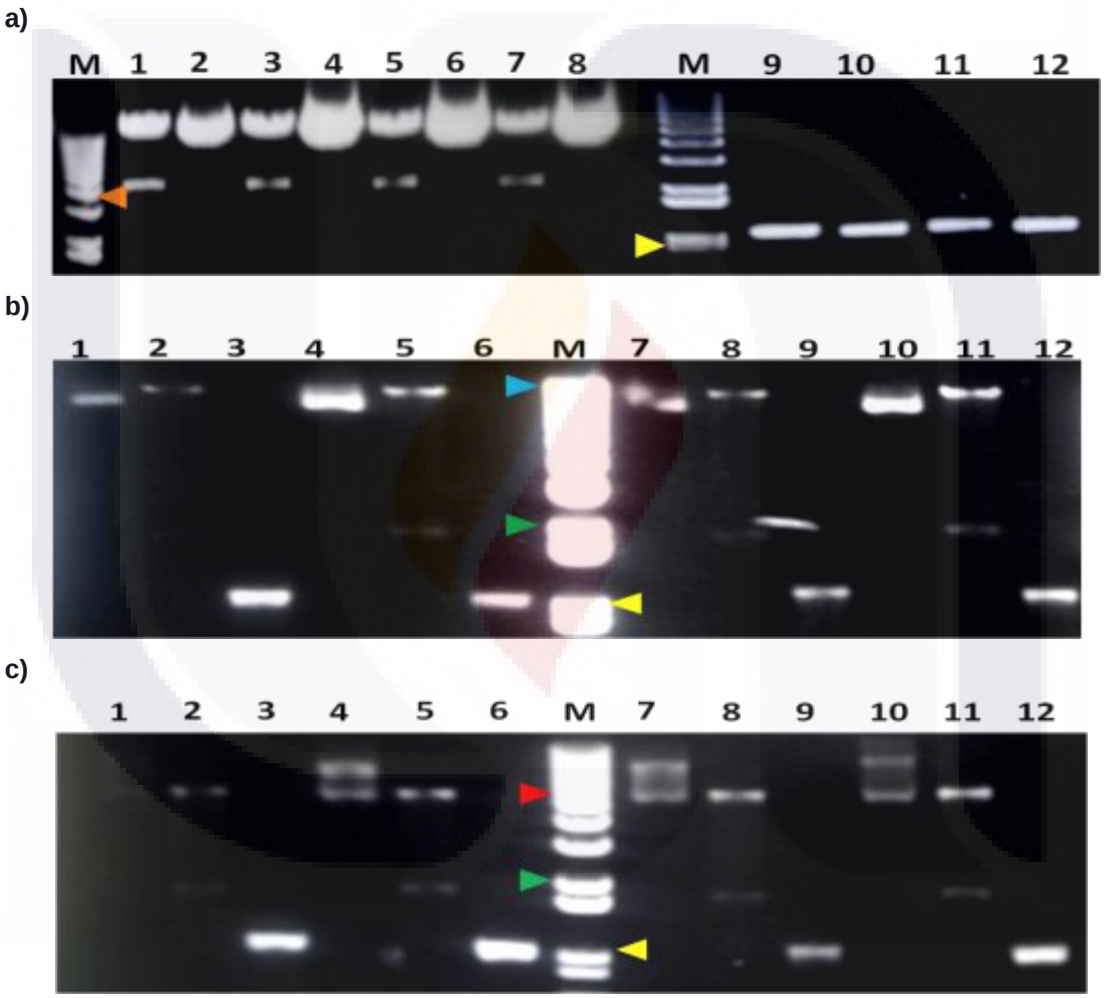
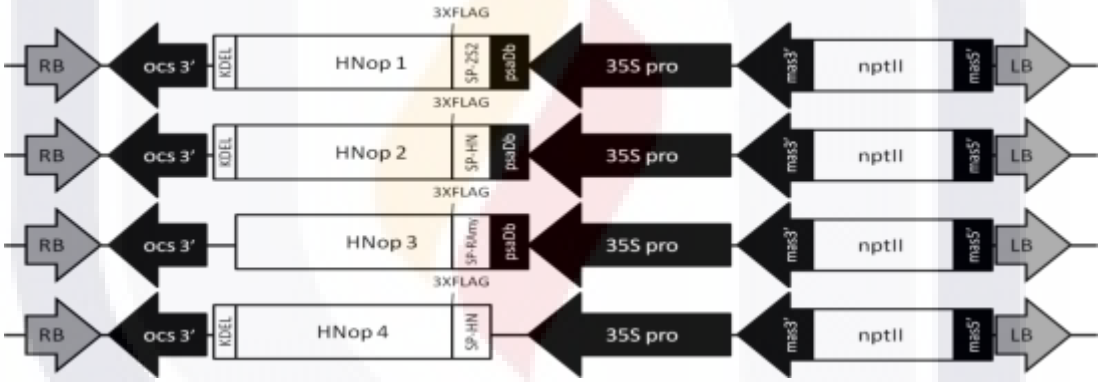


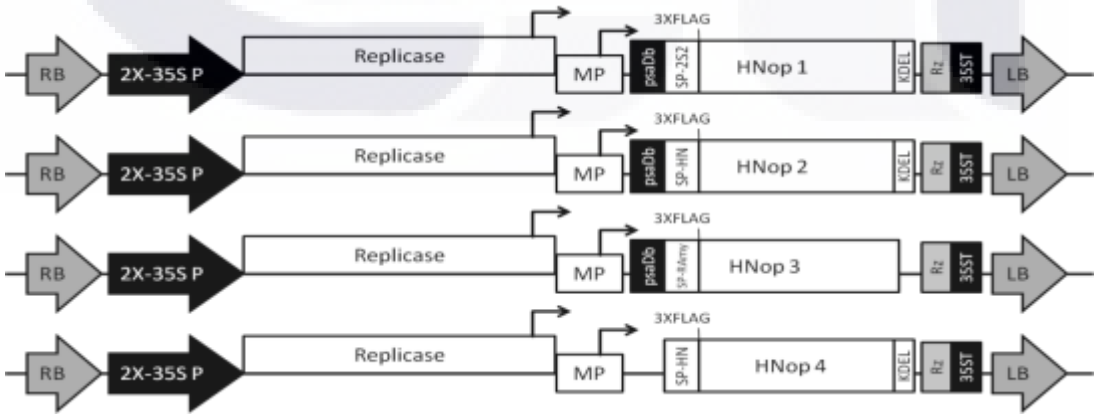
Figure 6.3 Testing of binary vectors with the HNop constructs. Plasmid DNA from single *E. coli* colonies, carrying the binary vectors with the HNop constructs was used to be tested through DNA digestion (Annex A, Protocol 1) and colony PCR (Annex A, Protocol 4). Restriction enzymes and primers used in the testing correspond to the same used during cloning work. **a)** Electrophoresis of plasmid DNA of p35S binary vector digested with AscI and amplified with CaMV35S-2 and HNop primers (Table 5.1). M, 1 kb Plus DNA Ladder (Invitrogen cat#10787-018); Lanes 1, 3, 5 and 7, p35S:HNop1, p35S:HNop2, p35S:HNop3 and p35S:HNop4 binary vectors digested with AscI, respectively (bands of ~4kb mean HNop construct + 35S promoter and ocs terminator); Lanes 2, 4, 6 and 8, non-digested p35S:HNop1, p35S:HNop2,

p35S:HNop3 and p35S:HNop4 binary vectors, respectively; Lanes 9-12, PCR product (~1kb) amplified from p35S:HNop1, p35S:HNop2, p35S:HNop3 and p35S:HNop4 binary vectors, respectively. **b)** Electrophoresis of plasmid DNA of pTRBO binary vector digested with PacI and AvrII and amplified with TRBO-1 and HNop primers (Table 5.1). M, 1 kb Plus DNA Ladder; Lanes 1, 4, 7 and 10, non-digested pTRBO:HNop1, pTRBO:HNop2, pTRBO:HNop3 and pTRBO:HNop4 binary vectors, respectively; Lanes 2, 5, 8 and 11, pTRBO:HNop1, pTRBO:HNop2, pTRBO:HNop3 and pTRBO:HNop4 binary vectors digested with PacI and AvrII, respectively (bands of ~2kb mean the HNop construct); Lanes 3, 6, 9 and 12, PCR product (~1kb) amplified from pTRBO:HNop1, pTRBO:HNop2, pTRBO:HNop3 and pTRBO:HNop4 binary vectors, respectively. **c)** Electrophoresis of plasmid DNA of pCMVva binary vector digested with PstI and HindIII and amplified with pMCSdelQAN-1 and HNop primers (Table 5.1). M, 1 kb Plus DNA Ladder; Lanes 1, 4, 7 and 10, non-digested pCMVva:HNop1, pCMVva:HNop2, pCMVva:HNop3 and pCMVva:HNop4 binary vectors, respectively; Lanes 2, 5, 8 and 11, pCMVva:HNop1, pCMVva:HNop2, pCMVva:HNop3 and pCMVva:HNop4 binary vectors digested with PstI and HindIII, respectively (bands of ~2kb mean the HNop construct); Lanes 3, 6, 9 and 12, PCR product (~1kb) amplified from pCMVva:HNop1, pCMVva:HNop2, pCMVva:HNop3 and pCMVva:HNop4 binary vectors, respectively. Yellow, green, orange, red, and blue arrows indicate the marker band size: 1kb, 2kb, 4kb, 6kb, and 10kb respectively.

a)



b)



c)

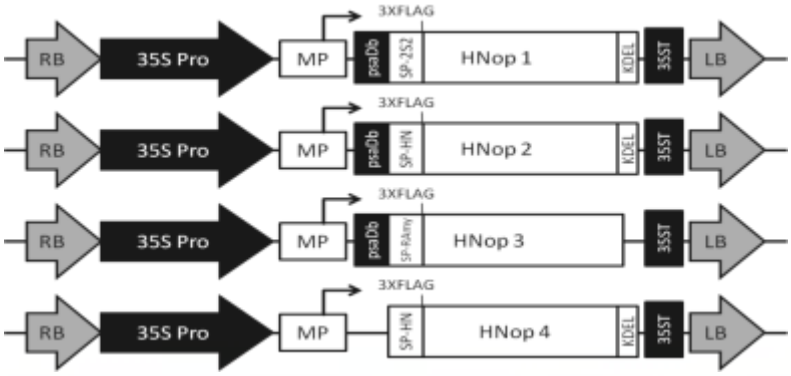


Figure 6.4 Maps of the T-DNA region of engineered binary vectors. Each HNop construct (HNop1, HNop2, HNop3 and HNop4) was cloned into the different binary vectors. **a)** T-DNA region of p35S binary vector carrying each HNop construct. **b)** T-DNA region of pTRBO binary vector carrying each HNop construct. **c)** T-DNA region of pCMVva binary vector carrying each HNop construct. Untranslated sequences are in bold and open reading frame (ORF) are indicated by white boxes. Arrowed boxes denote the orientation of coding region. Bent arrows mean subgenomic promoters. 35S Pro, cauliflower mosaic virus (CaMV) 35S promoter; 2X-35S P, CaMV duplicated 35S promoter; 35ST, CaMV polyA signal sequence/ terminator; 3XFLAG, fusion protein sequence; KDEL, endoplasmic reticulum retention signal; mas 5', *Agrobacterium* mannopine synthase unstralated 5' leader; mas 3', *Agrobacterium* mannopine synthase unstralated 3' terminator; MP, movement protein; ocs 3', *Agrobacterium* octopine synthase polyA signal sequence/terminator; nptII, neomycin phosphotransferase gene; psaDb, untranslated 5' sequence (5'UTR) of psaDb gene from *Nicotiana sylvestris*; Replicase, TMV 126K/183K ORF; SP-HN, signal peptide of HN protein's first 48 N-term aminoacids; SP-2S2, signal peptide of storage albumin from *Arabidopsis thaliana*; SP-RAmy, signal peptide of rice alpha-amylase; RZ, ribozyme. Right border (RB) and left border (LB) are represented by gray arrowed boxes.

6.2 TRANSIENT EXPRESSION OF HN PROTEIN IN *NICOTIANA BENTHAMIANA* LEAVES.

6.2.1 Growth of *Nicotiana benthamiana* plants.

N. benthamiana TW16 seed were planted and grown in greenhouse conditions, which included a 16-h photoperiod, temperature range of 75-85°F (25-30°C), a drip irrigation system and Osmocote fertilizer. Once seedlings were 2 weeks old, they were transplanted to a 6-inch pot (three per pot) and kept in same conditions for 3-4 weeks more. Upper leaves of 5-6 weeks old plants were used in all the experiments (Figure 6.5).

Figure 6.5 *N. benthamiana* plants grown in greenhouse conditions (UC Davis facilities). Plants of different age are being watered through a drip irrigation system.

6.2.2 Vacuum-agroinfiltration of harvested leaves.

Harevested *N. benthamiana* leaves (5-6 weeks old) were vacuum-agroinfiltrated with the different agro solutions cultured, harvested and resuspended in suspension buffer as described previously (Figure 5.8). Once the infiltrated leaves were removed from the 50ml tubes with the agro solution, they were let in the laminar flow hood to dry them (Figure 6.6). After that, dried leaves were placed on the mesh inside of plastic containers, adapted as humidity chambers, until they were used for sampling.

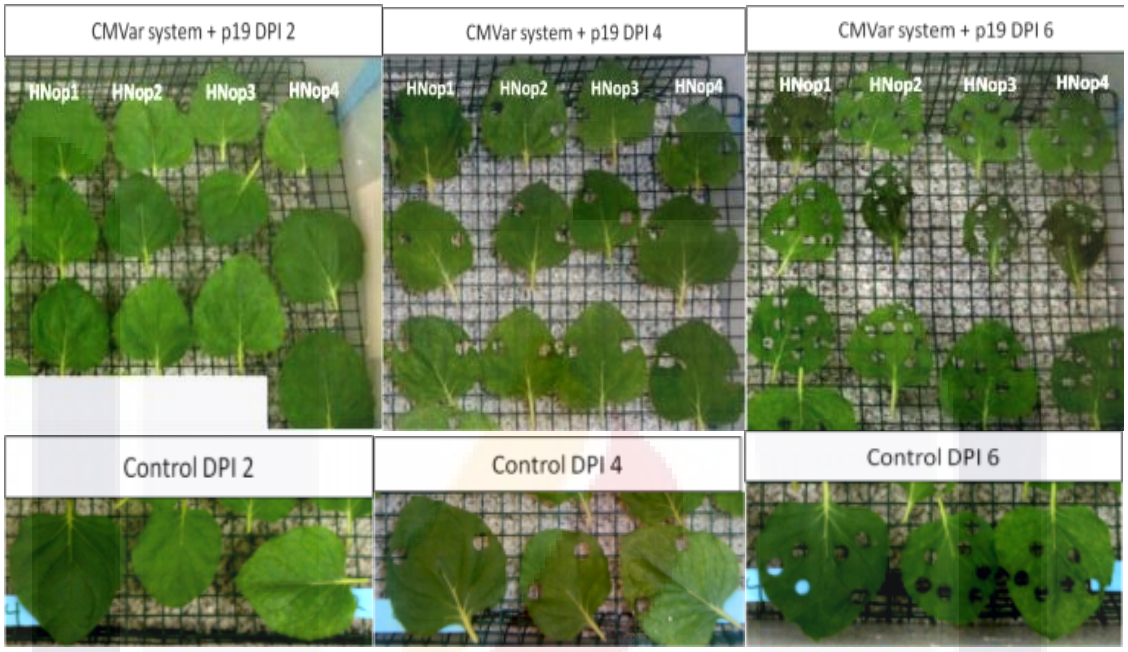
Figure 6.6 Harevested *N. benthamiana* leaves agroinfiltrated (left) and non-agroinfiltrated (right). Note that agroinfiltrated leaf looks wet and darker than non-agroinfiltrated leaves, because of the agro solution that has replaced the air located inside the plant tissues.

6.2.3 Sampling of agroinfiltrated leaves.

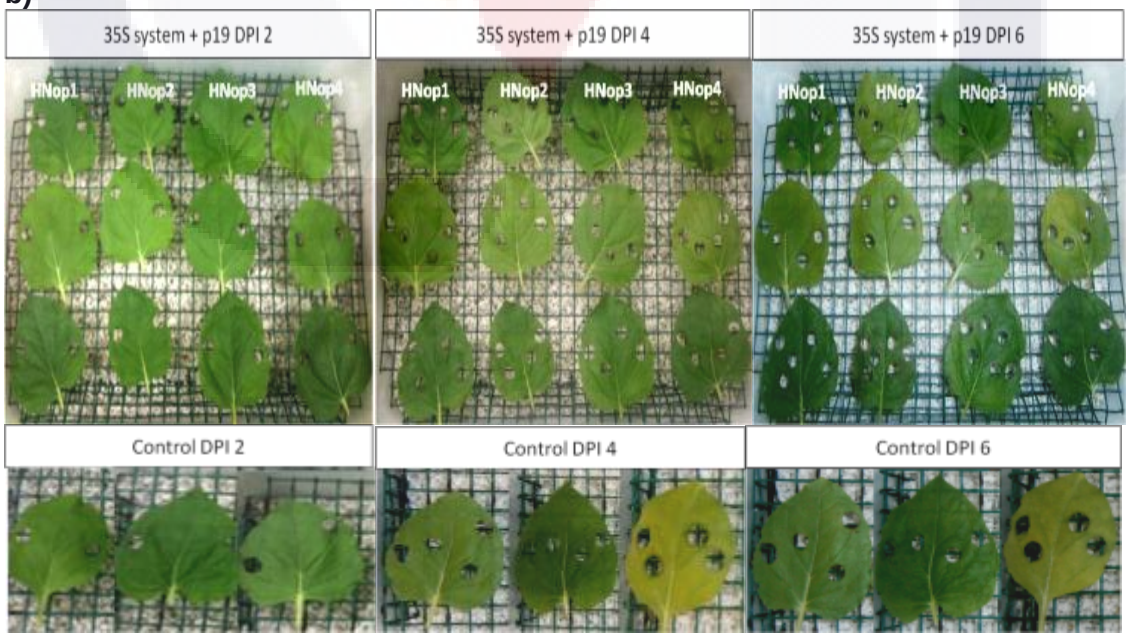
As described before, the sampling was achieved by punching leaf discs (~20mg) from agroinfiltrated leaves in second, fourth and sixth day post-infiltration (DPI), using the lid of a 1.5ml centrifuge tube per leaf. Cells were lysed with pre-cooled PBS buffer on ice,

using a sterile plastic pestle attached to a drill (Makita). Afterward, to compare two methods of extraction 100µl of cell lysed were sonicated for 20min at 4ºC and the rest does not. Then, they were clarified by centrifugation at 4ºC and 14,000rpm for 20 min. and the supernatant was collected (Figure 6.7) and immediately assayed for total soluble protein with the Bradford Assay. Supernatants were stored at −80ºC until required for ELISA and Western Blot analysis.

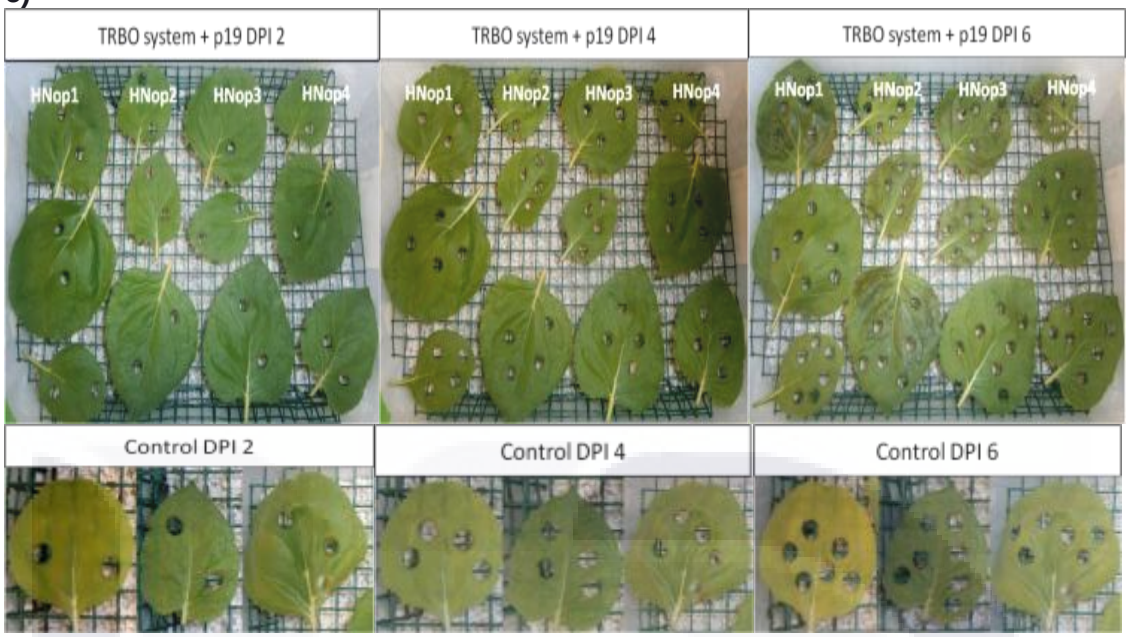
a)



b)



c)



d)

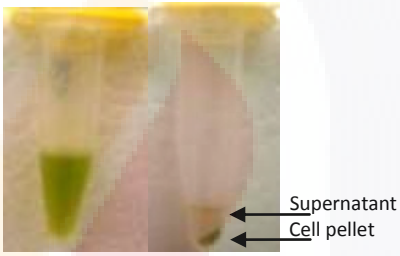


Figure 6.7 Sampling of agroinfiltrated leaves. Pictures show the incubation period of agroinfiltrated leaves with the different expression systems. The sampling was accomplished in second, fourth and sixth day post-infiltration (DPI). Three leaves were used per HNop construct either co-infiltrated or not with p19 gene silence suppressor, and for control (infiltrated with suspension buffer). **a)** Agroinfiltrated leaves using the CMVva system co-infiltrated with p19. **b)** Agroinfiltrated leaves using the 35S system co-infiltrated with p19. **c)** Agroinfiltrated leaves using the TRBO system co-infiltrated with p19. **d)** Cell lysed before (left) and after (right) clarification. The supernatant was used to perform quantitative and qualitative assays.

6.2.4 Bradford assay for total soluble protein (TSP)

In order to measure the production kinetics of protein expression in agroinfiltrated leaves, samples were taken from the leaves at the second, fourth and sixth day post-infiltration (DPI). Once the cell lysed was clarified, the supernatant of each leaf was immediately assay in triplicate and then kept at -80ºC until required for ELISA and Western blot. The kinetics of TSP expression of all the experiments are shown in figure 6.8. A decreasing TSP trend is observed in the three expression systems as well as in all HNop constructs and control within the three DPI. However, the TSP was only higher and significant different ($P<0.05$) in leaves agroinfiltrated with TRBO expression

system, with four HNop constructs, than in the control within DPIs (Figure 6.8; Annex C analysis 1). In DPI 2 HNop 1, 2, 3, and 4 reached 16, 16.7, 16.2, and 18.9 g/kg FW of TSP, respectively, meanwhile control expressed only 10.6 g/kg FW; in DPI 4 HNop 1, 2, 3, and 4 11.9, 13.7, 13.6, and 13.1 g/kg FW, respectively, meanwhile control expressed only 7.2 g/kg FW; in DPI 6 with the same trend HNop 1, 2, 3, and 4 reached 7.5, 8.9, 9, and 9.5 g/kg FW, respectively, meanwhile control expressed only 5.9 g/kg FW. In the case of leaves agroinfiltrated with 35S expression system, TSP was only higher and significant different ($P<0.05$) in DPI 2 than with TRBO and CMVva systems (Figure 6.8), and only HNop2 and HNop4 were significant different ($P<0.05$) than control (Annex C analysis 1). In DPI 2 HNop 1, 2, 3, and 4 expressed 23.4, 24.3, 24.7 and 27.2 g/kg FW, respectively, meanwhile control reached 20.5 g/kg FW; in subsecuent DPI 4 TSP decreased almost at half and in DPI 6 the TSP kept same. Finally, for leaves agroinfiltrated with CMVva expression sytem were significant different ($P<0.05$) with HNop1, 2 and 4 than control (Annex C analysis 1). In DPI 2 HNop 1, 2, 3, and 4 expressed 10, 10.1, 9 and 10 g/kg FW, respectively, meanwhile control reached 9.3 g/kg FW; for DPI 4 and 6 expression kept very similar, it only decreased in DPI 6. Additionaly leaves used for CMVva system had the least TSP and the TSP remained relatively stable during incubation.

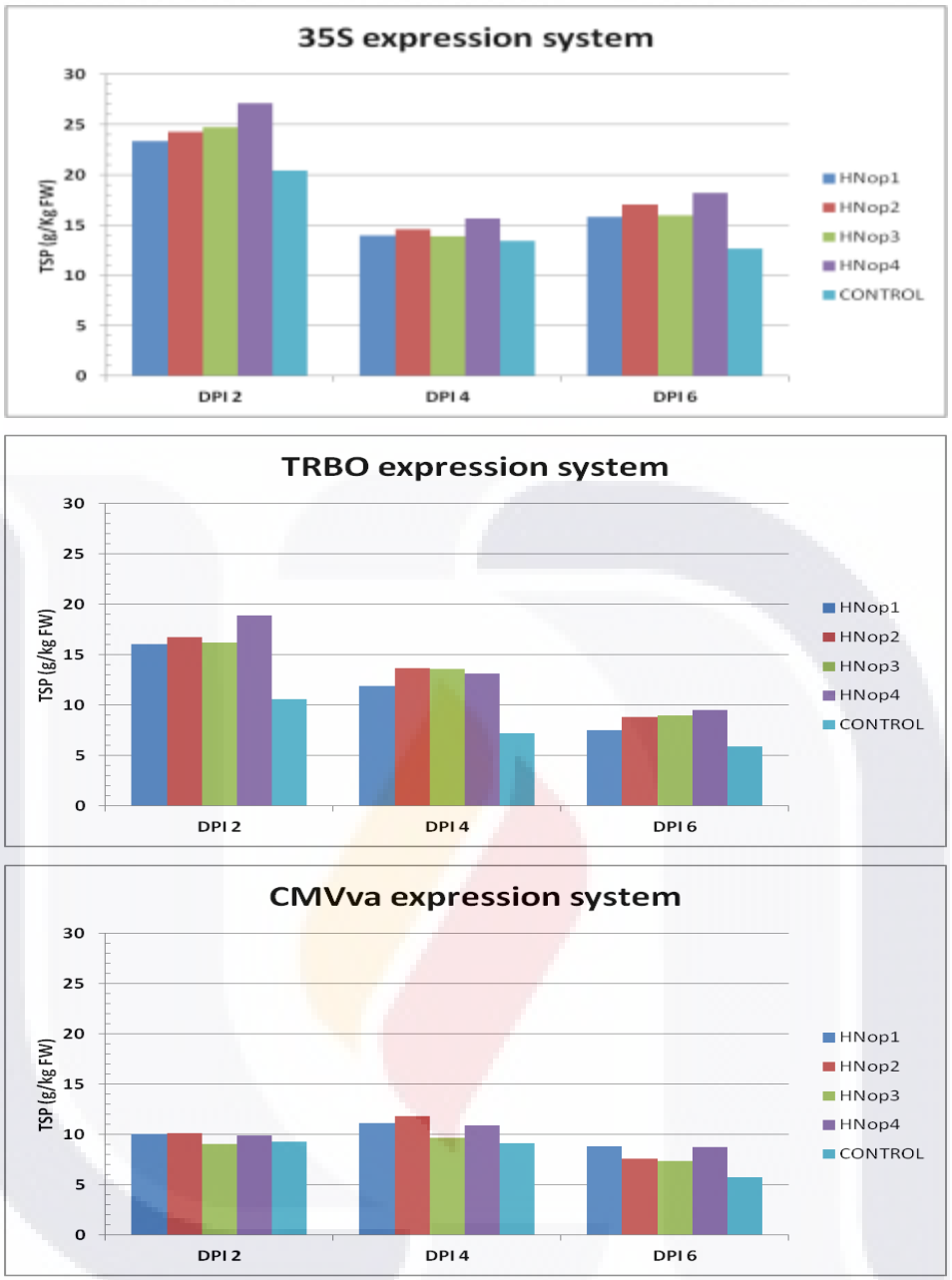


Figure 6.8 Measurement of total soluble protein (TSP) expression, through Bradford assay, in leaves agroinfiltrated with three different expression systems, co-infiltrated with p19 gene silence suppressor and sonicated. Decrese trend is observed in three expression systems with four HNop constructs as well as in control, within three DPIs (2, 4 and 6). TSP is represented as grams per kilogram of fresh weight (g/kg FW) and indicates the average of two separated experiments from triplicate assays performed on samples of triplicate *N. benthamiana* leaves. DPI= day post-infiltration.

6.2.5 ELISA assay and percentage of total soluble HN protein.

After measuring the TSP by Bradford assay, the supernatant of each sample was kept at -80ºC and then used to quantify, through ELISA assay, the amount of plant-expressed HN protein in agroinfiltrated leaves. Each sample was assayed in triplicated

and processed as described before (section 5.6.1). The accumulation of the plant-expressed HN was significantly higher at DPI 6 as well as higher levels were seen in leaves agroinfiltrated with TRBO system than with 35S and CMVva expression systems, and HNop1 construct was signiflicanty better than the rest. In addition, it was found a significant interaction not only between the expression systems with the HNop construct, but also with the DPI (Annex C). The major accumulated protein was accomplished using TRBO:HNop1 at DPI 6 (~20mg/kg FW) twice more than 35S and CMVva with any HNop construct (5-10mg/kg FW) (Figure 6.9).

Once HN protein concentration was determined, the values indicated as mg/kg of fresh weight (Figure 6.9), were divided between the amounts of TSP obtained in the last section and multiply for 100 to get the percentage of HN protein present in the total soluble protein, expressed in agroinfiltrated leaves in three different days post-infiltration (DPI 2, 4 and 6) (Figure 6.10). The plant-expressed HN protein represented as % of TSP was higher using TRBO expression system with the four HNop constructs than 35S and CMVva systems, during three DPIs. For the TRBO system a clear trend toward increasing HN protein expression was observed during DPIs, especially in HNop1 (Figure 6.10). At 2 DPI HNop 1, 2, 3 and 4 reached 0,074, 0.030, 0.041, 0.013 (% of TSP), respectively; at 4 DPI HNop 1, 2, 3 and 4 expressed 0.122 0.064, 0.081, 0.073 (% of TSP), respectively; at 6 DPI HNop 1, 2, 3 and 4 reached 0.237, 0.173, 0.160, 0.130 (% of TSP), respectively. In the case of the CMVva system a similar trend toward high HN protein expression was observed during DPIs, but only in HNop1 and HNop2 (Figure 6.10). At 2 DPI HNop 1, 2, 3 and 4 reached 0.045, 0.046, 0.039, 0.040 (% of TSP), respectively; at 4 DPI HNop 1, 2, 3 and 4 expressed 0.069, 0.086, 0.045, 0.049 (% of TSP), respectively; at 6 DPI HNop 1, 2, 3 and 4 reached 0.099, 0.153, 0.052, 0.050 (% of TSP), respectively. Finally using 35S system trend toward high HN protein expression was also observed, especially as with TRBO and CMVva, in HNop1 (Figure 6.10). At 2 DPI HNop 1, 2, 3 and 4 reached 0.022, 0.018, 0.017, 0.016 (% of TSP), respectively; at 4 DPI HNop 1, 2, 3 and 4 expressed 0.051, 0.039, 0.039, 0.040 (% of TSP), respectively; at 6 DPI HNop 1, 2, 3 and 4 reached 0.068, 0.043, 0.045, 0.045 (% of TSP), respectively.

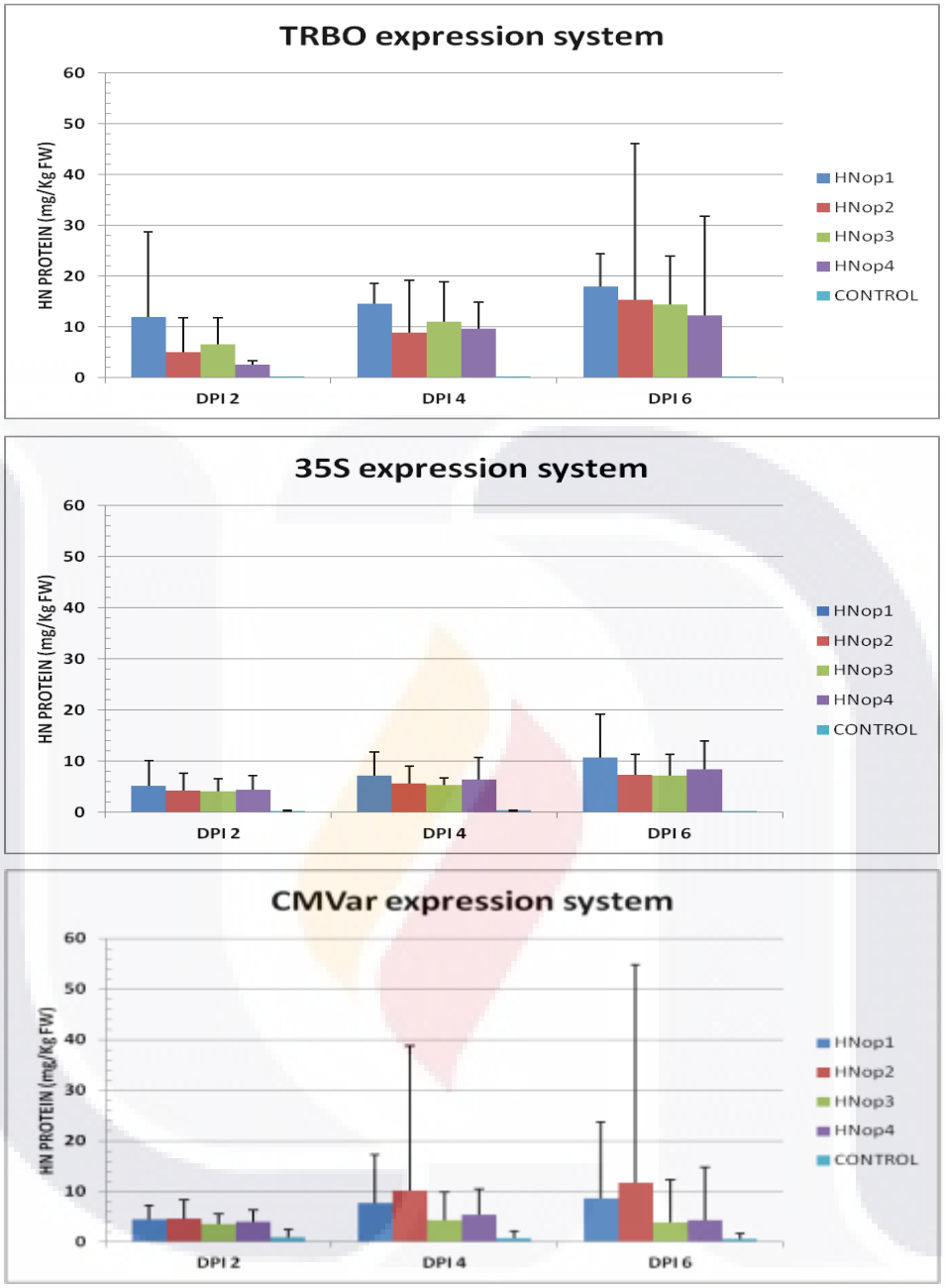


Figure 6.9 Concentration of plant-expressed HN protein in agroinfiltrated leaves with three different expression systems, co-infiltrated with p19 gene silence suppressor, and sonicated. The values are indicated as mg/kg of fresh weight (mg/kg FW) and represent the average of two separate experiments. Error bars are based on the propagation of errors calculated from triplicate assays performed on samples of triplicate *N. benthamiana* leaves. DPI= day post-infiltration.

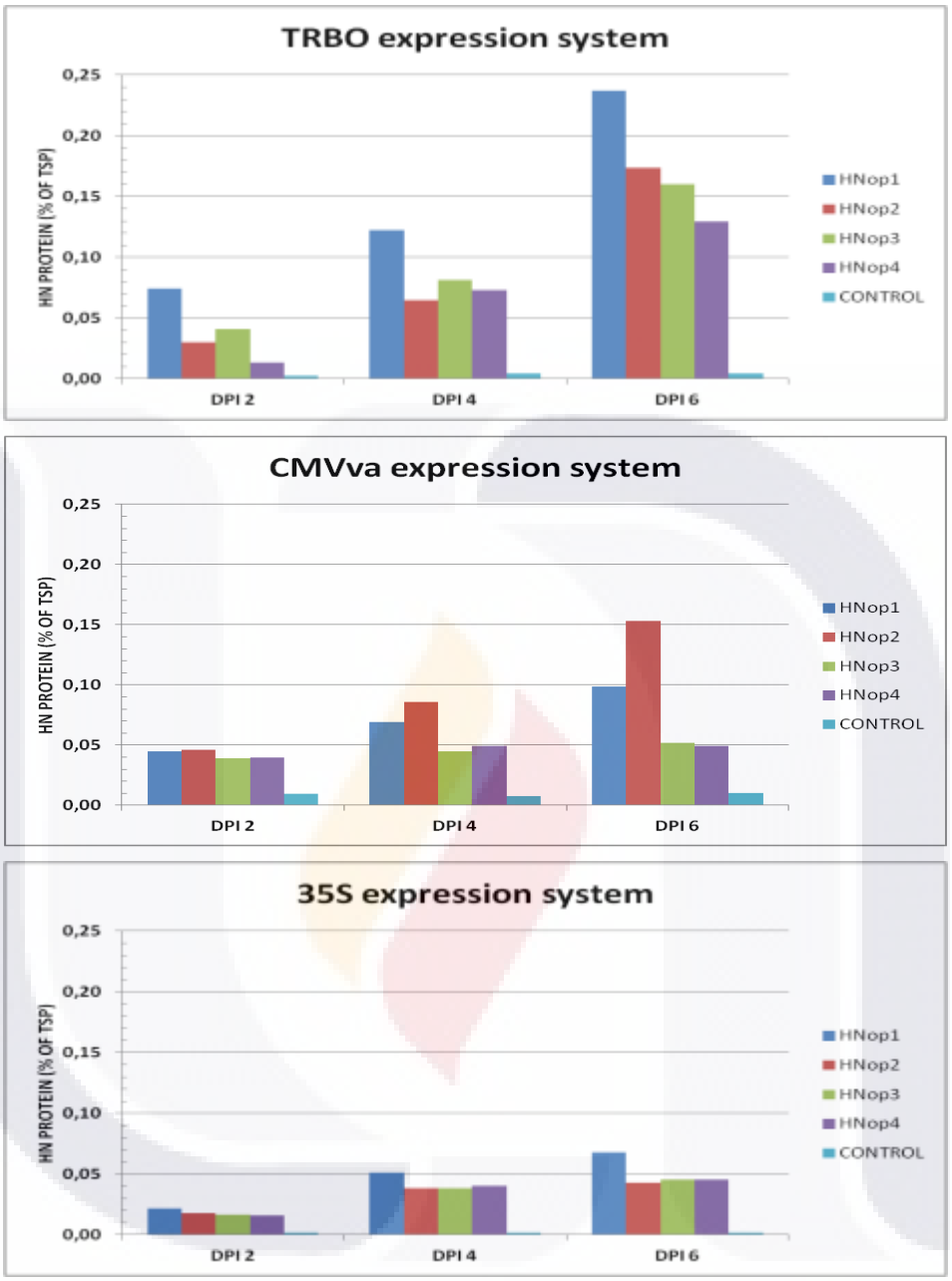


Figure 6.10 Percentage of HN protein in total soluble protein (TSP) in leaves agroinfiltrated with three different expression systems, co-infiltrated with p19 gene silence suppressor, and sonicated. The values are indicated as percentage of HN protein present in the TSP measured by Bradford assay and represent the average of two separated experiments from triplicate assays performed on samples of triplicate *N. benthamiana* leaves. DPI= day post-infiltration.

6.2.6 Western blot assay

Denatured SDS-PAGE was accomplished to separate proteins based on their molecular weight. Once achieved, proteins were transferred to a 0.45µm nitrocellulose membrane and immune detection was made using mAb anti-FLAG-HRP, followed by

visualization with TMB stabilized substrate. As a result, specific bands corresponding to plant-expressed HN protein using the different expression systems and a FLAG-BAP protein as a control are shown in figure 6.11. The band size expected for each HNop construct was ~60 kDa, which can be seen in all HNop constructs used within the three expression systems. In some cases an upper band can be observed (~120 kDa), which corresponds to a dimer of the HN protein. Meanwhile, the band size expected to positive control (FLAG-BAP) was ~50 kDa, which can be noted clearly (Figure 6.11).

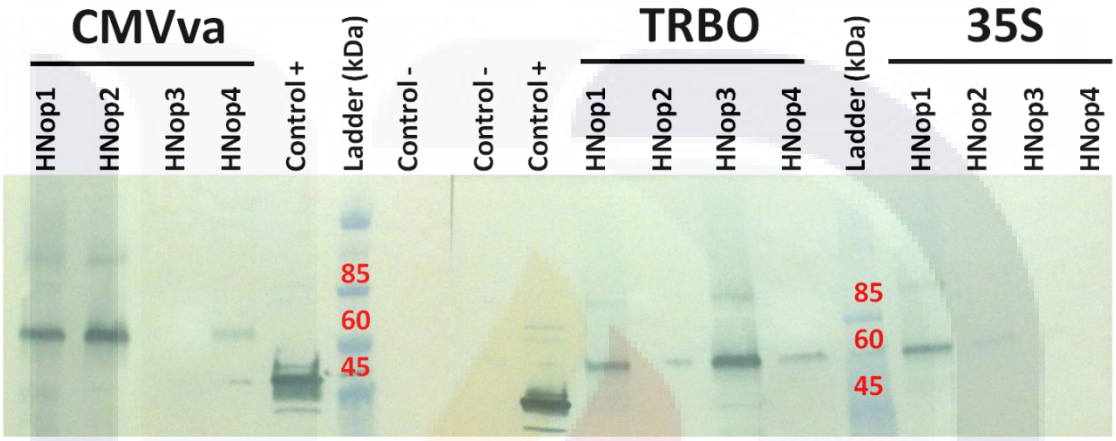


Figure 6.11 Western blot analysis of agroinfiltrated *N. benthamiana* leaves with the different expression systems (TRBO, 35S and CMVva). As positive control (Control +) the FLAG-BAP protein was used and as negative control (Control -) a leave infiltrated with suspension buffer. In HNop lanes, bands around 60kDa were considered as positive, and in control + lane the band around 50kDa. Upper bands (~120kDa) were considered as HN dimers. Loaded samples come from DPI 6 co-infiltrated with p19 gene silence suppressor and sonicated. The same volume was loaded for each sample.

7. DISCUSSION

Selection of NDV strain. Based on recent outbreaks worldwide, it seems that emergence of new velogenic NDV strains is related to the influence of antibody immune selective pressure, which induce antigenic variation, given for intensive vaccination programs with the same strain, mostly genotype II (i.e. La Sota strain) (Cho et al 2008; Gong and Cui 2011; Gu et al 2011). With this approach, Cho et al (2008) demonstrated the importance of the variation in the HN protein's linear epitopes from the Korea strains. They synthesized three oligopeptides from the linear epitope (346-358 amino acids) region. Two of them had one or two mutations (E347K; E347K and M354K) and the other was the common epitope sequence. They found that anti-La Sota chicken antiserum reacts less strongly to oligopeptides with mutations than with the common epitope sequence. Following a similar strategy, Gu et al (2011) used

recombinant flowpox viruses expressing HN genes from a Chinese field strain and La Sota strain. They found that chicken anti-serum from chickens vaccinated with recombinant La Sota vaccine, reacts poorly with the Chinese field strain, which has mutations in V266A, E347K and T540A. Subsequently, Gong and Cui (2011) performed, in chicken embryo fibroblast cells, a selective pressure with anti-serum of a field strain. The genetic mutations predominantly occurred in the 20th–30th generations, where three non-specific mutations in the HN gene were related to immune selective pressure (aa# 353, 521 and 568) and were located exactly within two of the three known antigenic epitopes: 346-353 and 512-521 (Iorio et al 1991). In the case of mutations in the epitope region 346-352 (E347 or R353), seems to be the more conservation or consistant mutation. In addition, results described above are very consistent with those previously reported by Miller et al (2007), where homologous strains seem to protect better than heterologous strains. In our case, we chose the HN sequence from a velogenic strain isolated from last outbreak in California (CA02), whose potential as vaccine has been proved previously (Miller et al 2007). What we found when performed a multiple alignment of different NDV genotypes, was that CA02 has mutations in I352V and S520T (Figure 6.1 [7]) similar to Mexican strains (Figure 6.1 [8 and 9]) which was expected since all of them belong to the genotype V. Even though antigenic variations were not the same in CA02 than reported before (Cho et al 2008; Gong and Cui 2011; Gu et al 2011), they are present within the epitope regions, suggesting that differences in protection of CA02-vaccinated chickens found by Miller et al (2007) against homologous and heterologous NDV strains is likely related with the antigenic variation found in this study for CA02 strain. Moreover, mutations found by authors above, were present in Asiatic strains that belong mostly to genotype VII, which can explain those difference in mutation sites found in this study. In addition, it is well known that the majority of genomic changes in non-segmented RNA viruses are due to either the intrinsic error rate of the polymerase or as a result of recombination (Miller et al 2009). However, the influence of antibody immune selective pressure on NDV HN gene appears to be likely, as Asiatic strains, to the intensive vaccination programs made with the same strain, because of the most of commercial vaccines in the US and Mexico are formulated with La Sota strain (USDA 2011; SAGARPA 2012). On the other hand, it is thought that antigenicity of the HN protein is not only determined by the epitope regions, but also by the protein maturity and molecule conformation (James et al 2000). Those features are mainly determined by proper folding and the maintenance of its three-dimensional structure through disulfide bonds between cysteine residues, which are conserved in HN peptide and located at aa# 172,

186, 196, 238, 247, 251, 344, 455, 461, 465, 531 and 542. Thus, a specific mutation in at least one of these residues could potentially block the correct folding of the three-dimensional structure (Sagrera et al 2001). Such cysteine residues did not mutate in CA02 (Annex B Table 1), suggesting that mutations under immune selective pressure only occur in given epitopes, rather than the entire three-dimensional structure of the molecule. Additionaly, N-glycosilation plays an important role in the antigenicity of the HN protein and virulence of NDV (Panda et al 2004), although animals and plants cells have different patern glycosilation, tobacco and potato-expressed HN protein has already been tested as vaccine giving similar and further protection compared with a commercial NDV vaccine (Berinstein et al 2005; Hahn et al 2007). Therefore, is expected that the plant-expressed HN protein in this study should protect birds like the commercial vaccines, with no side effects. However, *in vivo* analysis are required to agree with reported by the authors.

Codon optimization and engineering of HNop constructs. Synthetic gene production is an enabling technology for improved protein production, since it can be used to produce totally novel gene sequences, which are optimized for codon usage and other sequence features anticipated to facilitate improved protein expression in defined expression systems (Stewart 2007; Jung and McDonald 2011). Therefore, to boost HN expression levels, as described before (Section 5.1.2) HN cDNA was engineered through addition of sequences as well as codon optimization that will be enhance HN expression level. Even though, all additional sequences to the native HN cDNA were inserted to improve the HN expression, codon optimization plays an important role in this approach. Since rare codons have been well-documented to have a negative impact on protein expression in *E. coli* and other organisms (Stewart and Burgin 2005), one of the primary applications of complete gene synthesis is to reengineer the gene so that its codon bias more closely matches that of the intended heterologous expression host, in our case *N. benthamiana.* With this approach, the optimized HN (HNop) constructs were analyzed in terms of Codon Adaptation Index (CAI), Effective number of codons (Nc), and guanine and cytosine (GC) content as shown in table 6.1. The CAI ranges from 0 to 1 where higher CAI means highly codon biased or higher codon usage similarity between two different codon usage tables (Sharp and Li 1987). Nc is a number between 20 and 61 where 20 means extremely biased and 61 stands for equally biased between synonymous codons (Wright 1990). GC content variation is the most important parameter differentiating codon bias between different organisms, where prokaryotic organisms are GC-rich, meanwhile

eukaryotic organisms are AT-rich (Chen et al 2004). Nc was kept very similar from the original, only increasing in HNop1 from 56.09 to 58.17 and decreasing around 1.5 in the rest (Table 6.1), which means that the optimized gene had less biased codon usage. CAI was not too much adjusted from the original 0.68 to 0.72-0.75 in all HNop constructs, which accord with Nc values as well as GC content at the third codon letter kept like from original ~45.5 to 44.5 % (Table 6.1) expecting formation of weak mRNA secondary structures. These results, likely are due the fact that virus genomes have evolved to be transcript and translated in eukaryotic cells, mimicking host's codon bias as strategy. This has already reported for a member of paramyxovirus family (Liu et al 2011), with similar GC content to the NDV HN. In addition, synthetic gene design allows one to use synonymous nucleotide changes to 'silently' eliminate undesired MCS restriction endonuclease sites from the body of the ORF (Stewart 2007; Jung and McDonald 2011). Therefore, to take full advantage of the utility of multiple cloning sites of the three expression systems used in this study (TRBO, CMVva and 35S), undesired restriction site were removed as well as other undesired sequences such as repeated DNA sequence, potencial polyadenylation signal, and potencial polyadenylation signal (Stewart 2007; Jung and McDonald 2011) were reduced, also there was different percentages of changed bases and codons to each HNop construct (See Annex B analysis 1), which means HNop constructs must be expressed without transcriptional or translational problems.

Comparison of HNop constructs. As described previously in figure 6.2, four HNop constructs were synthesized with different elements with a goal to achieve high HN protein levels in harvested *N. benthamiana* leaves. In order to give the same chance to each binary vector to be transferred into plant cell, vacuum agroinfiltration was performed as described before (Arzola et al 2011; Simmons et al 2009; Plesha et al 2009; Sudarshana et al 2006; Gleba et al 2005; 2007; Marillonnet et al 2005) with little modifications (See figure 5.8). When leaves looked like wet (Figure 6.6), which means agrobacteria solution was correctly introduced into the intercellular space, they were considered as agroinfiltrated. Also leaves health was considered during experiments, they kept helthy within DPIs (Figure 6.7) that agree with reported previously for vacuum-agroinfiltrated harevested *N. benthamiana* leaves (Arzola et al 2011; Plesha et al 2009). About HNop constructs elements, addition of 5'UTR sequences upstream of of the transgene has already shown its effectiveness increasing heterologous protein production in plants (Carrington and Freed 1990; Turner and Foster 1995; Yamamoto et al 1995; Zeenko and Gallie 2005; Joensuu et al 2008). Specifically, in our case the

5‘-leader sequence of *psaDb*, which is a nuclear gene for a photosystem I subunit in *N. sylvestris* (Yamamoto et al 1995), has been used to produce a recombinant anthrax receptor fusion protein (CMG2-Fc), reaching up to 0.56 g per kg of leaf fresh weight after 3.5 days postinfiltration (Arzola et al 2011). Among the four HNop constructs, only HNop4 does not have the psaDb enhancer. Even though, expression levels measured by ELISA assays at DPI 2, 4 and 6 between HNop4 and HNop3 were not significantly different ($p>0.05$) within the three expression systems (Figure 6.9; 6.10 and Annex C), only in the 35S system HNop4 was not significantly different than HNop2 and HNop3 within three DPIs (Figure 6.9; 6.10). Since all HNop constructs were co-infiltrated with the viral silencing RNA suppressor (VSR) p19, whose suppressor activity has been confirmed elsewhere (Voinnet et al 2003; Qu and Morris 2002; Takeda et al 2002; Plesha et al 2009; Arzola et al 2011), it is likely that p19 has a compensatory action that in some way balances the lack of a enhancer in HNop4, which could explain why HNop4 reached similar expression levels compared to HNop3 and HNop2 in the 35S system and also compared with HNop3 in TRBO and CMVva systems.

On the other hand, HNop1, 2 and 4 proteins were targeted to the ER lumen and labeled with a retention signal (KDEL) (Figure 6.2) because it is the most promising subcellular compartment for the proper folding and assembly of complex recombinant eukaryotic proteins in plants (Fischer and Emans, 2000), and many reports have demonstrated higher accumulation of secreted recombinant proteins in this intracellular compartment (Arzola et al 2011; Conley et al 2009; Joensuu et al 2008; Fiedler et al., 1997; Huang et al., 2001; Menassa et al., 2001; Ramirez et al., 2002; Wandelt et al., 1992); and HNop3 was targeted to the apoplast (Figure 6.2), which is known to be poor in terms of protease activity and keeping the integrity of the recombinant protein (Moloney and Holbrook 1997). To achieve the targeting to both subcellular compartments, the signal peptide is crucial in this approach. Comparing the yield of all the HNop constructs, it is seems that the signal peptide was a determinant factor, due to the fact that the S2S signal peptide (HNop1) corresponded to higher levels and more consistence between three DPIs and expression systems (Figure 6.9 and 6.10). Another interesting point about the higher yields of the HNop1 construct, it is the fact that it had higher values of Nc during codon optimization (Table 6.1), which might also explain some of the differences. Although HNop2 had more percentage of HN in terms of the TSP using CMVva system (Figure 6.10), the variation seen in ELISA assay (Figure 6.9) indicate that this system may not be as reproducible. In addition, it has already been reported that the native HN signal peptide had better expression level

than a heterologous signal peptide (Gomez et al 2009). However, in this study it was found that the construct with 2S2 signal peptide (HNop1) was significantly better ($p<0.05$) than constructs with native HN (HNop2 and 4) and Ramy signal peptide (HNop3) in the three expression systems (Figure 6.9; 6.10 Annex C). Finally, to quantify and to visualize the plant-made HN protein, a FLAG-tag sequence was added and accord with reported in the literature (Einhauer and Jungbauer 2001), it was very useful during performing of ELISA and Western blot assays, as well as should also make purification easier.

Comparison of 35S, TRBO and CMVva expression systems. Since the last decade, plant virus-based expression vectors have developed further for use as effective vaccine and therapeutic protein production systems (Yusibov et al 2011). Hence, to evaluate the effect of using virus-based or non-viral vectors, HNop constructs were inserted within pTRBO, pCMVva (viral-based vectors), and p35S (non-viral vector) binary vectors (Figure 6.4) and tested to verify their properly position (Figure 6.3). The experimental results are shown in figure 6.9 and 6.10 as well as statistical analisys can be seen more detailed in Annex C. Important results were found, because of all the systems expressed HN protein throughout the incubation period. Thus, it was expected to obtain more protein concentration in DPI6 than at the other DPIs as it has been reported (Plesha et al 2006; 2009; Arzola et al 2011; Werner et al 2011). It was exactly we found; at 6 DPI recombinant protein levels were significantly different ($p<0.05$) than DPI4 and 2 in all expression systems. In addition, not only a significant interaction ($p<0.05$) between expression system and DPI was found, but also between expression system and HNop constructs (Annex C). To be specific, with TRBO the average of expression level of HN was about 12~18 mg/kg fresh weight (FW); in the case of CMVva the average was 4~8 mg/kg FW; meanwhile for 35S 7~11 mg/kg FW. Thus, TRBO system that has shown one of the highest expression levels in plants (Lindbo 2007) gave the best results in this study. However, expression reported for Lindbo was much higher (up to 5.5 g/kg FW of GFP); for this reason is important to realize that not every protein expressed from a viral vector will necessarily accumulate to very high levels, there are other effects involved in the final accumulation level of any protein such as protein stability. Intersting, the TRBO system is driven by the constitutive CaMV 35S promoter like CMVva and 35S expression systems, however, TRBO has a duplicate version of this promoter, which could explain in part the high level obtained with this system (Figure 6.4). On the other hand, the CMV-based system has shown to give great expression level of α-1antitripsyn (AAT) when it was engineered as a

monopartite inducible viral amplicón (CMViva [Sudarshana et al 2006; Plesha et al 2009]). In addition, a tripartite version (CMVva) has also been developed and used to express an endonuclease either in *N. benthamiana* or sunflower, however expression level reported (US Patent application 20120045818-Plant-Based Production of Heterologous Proteins, Hwang et al 2012) was lower than with CMViva. These results agree with those seen in this study, since amount of HN protein was even lower than expressed with 35S. This could be explained due to the fact that CMVva is a tripartite expression system (Figure 5.6), thus, the success of this system only will be possible if the three RNAs are in the same cell at the same time. Even though the vacuum might overcome this issue, the probability of achieving it is much less than with a monopartite system such as TRBO or the monopartite CMViva. In addition this could explain the great variations observed whit the HNop constructs within the DPIs (Figure 6.9). Another important point is that the *A.tumefaciens* strain used by Plesha et al (2009) and Sudarshana et al (2006) to carry the CMViva system was EHA105:pCH32, the same that used for 35S and TRBO system in this study. On the other hand, the tripartite CMVva system was cloned in GV3101 strain, which could explain in part the low expression levels reached with the CMVva system compared with the CMViva system used by the autors. In the case of 35S expression system, it is known that high level recombinant protein production can be accomplished when co-infiltration with VSRs is carried out (Voinnet et al 2003; Qu and Morris 2002; Takeda et al 2002; Plesha et al 2009; Arzola et al 2011). Accordingly, in our case the HN protein level was a little more with 35S than with the CMVva expression system, although latter is a virus-based system that is expected to give more expression (Yusibov et al 2011). However, this difference could be to the lack of success of the CMVva compared with the use of the monopartite 35S system with a strong constitutive promoter.

Another interesting point, is the fact that with the CMVva system, specifically in HNop1 and HNop2, the percentage of HN in the total soluble protein (TSP) was higher (0.10~0.15%) than with 35S system (0.4~0.7%), although the TSP in the leaves used for CMVva had almost twice less than used for 35S (Figure 6.8), which shows the effieciency of virus-based systems even with lower TSP in the leaves. This has some advantages in terms of downstream processing since the starting material will have a higher level of target product compared with unwanted protein contaminants.

The results of this study demonstrated that transient expression performed with virus-based vectors and engineered genes, especially TRBO:HNop1, higher yields can be accomplished instead of using transgenic plants, which agrees with results reported

previously (Yusibov et al 2011; Tiwari et al 2009; Fischer et al 1999). For example, Beristein et al (2005 cited by Joensuu et al 2008), Hahn et al (2007) and Cardineau et al (US patent application US2008/0076177) had a yield of HN protein reported as 0.06% TSP in transgenic potato leaves; 0.069% TSP in transgenic tobacco leaves; and up to 1% TSP in transgenic tobacco cell lines, respectively. Which was similar to expressed by Beristein et al (2005) and Hahn et al (2007), using the 35S system, but up to 4-fold yield increase was obtained using TRBO:HNop1 system (0.23% TSP). In the case of Cardineau, although they produced 4 fold more protein; it is interesting to note that their cell culture system shares some of the same cost disadvantages of other bioreactor-based process (Table 1.1), and likely for this reason this system was never introduced into the market. Another strategy has been attempted, like displaying the epitope of 346-353 region of HN in CMV coat protein. Zhao and Hammond (2005) through transient expression of the modified CMV RNA3 reached up to 430mg/kg FW of coat protein carring the epitope. However, this strategy of displaying the epitopes could be not efficient in immune response, because amino acids that flank the epitope regions play an important role during recognition of epitopes, thus the lack of them or their mutation affects the antigenicity of the HN protein (Cho et al 2008). Currently, using a non-viral transient expression system and comparing five constructs with two different signal peptides, Gomez et al (2009) reported the production of full length HN protein in *N. benthamiana* leaves. Even though they could characterize the recombinant HN protein by qualitative Western blot, the yield achieved (3µg/mg of total leaf protein or 0.3% of TSP) was not clearly demonstrated, because they did not show any quantitative analysis (ELISA) to corroborate what they reported. Hence, it seems that the use of TRBO:HNop1 system has the best yield of plant-expressed HN protein so far, at least in plant tissue either transgenic or non-transgenic.

Western blots were used to characterize the product size and to assess the degree of proteolytic processing for all the samples as described in section 5.7.1. The expected size for all recombinant mature HN was 60.54 kDa. The molecular weight was hypothesized by Compute pI/MW tool (http://web.expasy.org/compute_pi/), and the observed MW was very close to what was expected (Figure 6.11). As reported in previous previously (Gomez et al 2009; Hahn et al 2007; Zoth et al 2011; US patent application US2008/0076177, Cardineau et al) the molecular weight of the plant-made HN was similar to the native HN (~70kDa), in our case the hipothesized molecular weight of the mature native HN (from 27 to 571 aa) was 59.56 kDa. It is important to note that hypothesized molecular weight does not consider the N-glycosylation that

occurs as posttranslational modification in eukaryotic cells, which has shown increase the molecular weight in plant-made HN protein (Gomez et al 2009) up to 74 kDa in HN NDV La Sota strain. Despite good full expressed HN protein in this study, it is important consider further analisys to identify the correct translation and N-glycosylation in the plant-made HN protein.

8. CONCLUSIONS

After the screening of expression systems, HNop constructs, and DPIs significance differences ($p<0.05$) were found. The highest yield was obtained at DPI 6 using the TRBO expression system carryng the HNop1 construct (~20mg/kg FW), validating the hypothesis that plant virus-based vectors can provide higher productivities. Through targeting the plant-expressed HN protein to the ER, higher protein accumulation was seen, especially with 2S2 signal peptide (HNop1). Most important, in this study we demonstrated that using the platform SwiftVax® in six days we can produce up to 1000-1300 doses of 15-20µg (dose used either in a mice (Berinstein et al [2005]) or chicken (Hahn et al [2007] model), and that future outbreaks can be overcome producing subunit vaccines in days instead of months. Thus, the production of plant-made NDV HN protein through this simple and cheaply technology, it could become a real market opportunity in developing countries, where most vaccines, either veterinary or human, are imported from developed countries, making it difficult to implement vaccination programs. Finally, although we have obtained good yields of HN protein, immunogenic tests are necessary to validate the antigenicity of our plant-made HN protein.

9. FUTURE DIRECTIONS

Image infiltrated *N. benthamiana* leaves with confocal microscopy to determine where within the leaf the HN is being expressed.

Perform co-infiltrations with different gene silencing suppressor to see effect on expression level.

Purify and characterize the plant-made HN protein to determine its N-glycosylation sites and glycoforms.

Test the HN protein as a vaccine *in vivo* to analyze its immunogenicity and possible side effects.

10. GLOSSARY

Affinity tag; purification tag An amino acid sequence that has been engineered into a protein

to make its purification easier. These can work in a number of ways. The tag could be another protein, which binds to some other material very tightly and thus allowing the protein to be purified by affinity chromatography (q.v.). The tag could be a short amino acid sequence, which is recognized by an antibody. The antibody would then bind to the protein whereas it would not have done so before. One such short peptide, called FLAG, has been designed so that it is particularly easy to make antibodies against it. The tag could be a few amino acids, which are then used as a chemical tag on the protein. For example, a string of positively charged amino acids will bind very strongly to a negatively charged filter: this could be used as the basis of a separation system. Some amino acids bind metals very strongly, especially in pairs: this chemical property can be exploited by using a filter with metal atoms chemically linked onto it to pull a protein out of a mixture of proteins. *cf* affinity chromatography.

Agarose gel electrophoresis A process in which a matrix composed of a highly purified form of agar is used to separate larger DNA and RNA molecules. *See* electrophoresis.

Agrobacterium tumefaciens A bacterium that causes crown gall disease in some plants. The bacterium infects a wound, and injects a short stretch of DNA into some of the cells around the wound. The DNA comes from a large plasmid - the Ti (tumour induction) plasmid - a short region of which (called T-DNA, = **t**ransferred DNA) is transferred to the plant cell, where it causes the cell to grow into a tumour-like structure. The T-DNA contains genes which *inter alia* allows the infected plant cells to make two unusual compounds, nopaline and octopine, that are characteristic of transformed cells. The cells form a gall, which hosts the bacterium. This DNA-transfer mechanism is exploited in the genetic engineering of plants. The Ti plasmid is modified so that a foreign gene is transferred into the plant cell along with or instead of the nopaline synthesis genes. When the bacterium is cultured with isolated plant cells or with wounded plant tissues, the "new" gene is injected into the cells and ends up integrated into the chromosomes of the plant.

***Agrobacterium tumefaciens*-mediated transformation** A naturally occurring process of DNA transfer from the bacterium *A. tumefaciens* to plants.

Amplify To increase the number of copies of a DNA sequence, either *in vivo* by inserting into a cloning vector that replicates within a host cell, or *in vitro* by polymerase chain reaction (PCR).

Antibiotic A class of natural and synthetic compounds that inhibit the growth of or kill some micro-organisms. Antibiotics such as penicillin are often used to control (to some extent kill) contaminating organisms. However, resistance to particular antibiotics can be acquired through mutations. Some contaminating organisms are only suppressed or their metabolism slowed to an insignificant level. See antibiotic resistance; bactericide; bacteriostat.

Antibiotic resistance The ability of a micro-organism to produce a protein that disables an antibiotic or prevents transport of the antibiotic into the cell.

Antibody (Gr. anti, against + body) An immunological protein (called an immunoglobulin, Ig) produced by certain white blood cells (lymphocytes) of the immune system of an organism in response to a contact with a foreign substance (antigen). Such an immunological protein has the ability of specifically binding with the foreign substance and rendering it harmless. The basic

immunoglobulin molecule consists of two identical heavy and two identical light chains. See monoclonal antibodies; polyclonal antibodies.

Antigen; immunogen A compound that elicits an immune response by stimulating the production of antibodies. The antigen, usually a protein, when introduced into a vertebrate organism is bound by the antibody or a T cell receptor. See antigenic determinant; antigenic switching.

Antigenic determinant A surface feature of a micro-organism or macromolecule, such as a glycoprotein, that elicits an immune response. See epitope.

Attenuated vaccine A virulent organism that has been modified to produce a less virulent form, but nevertheless retains the ability to elicit antibodies against the virulent form.

Base pair (bp) The two strands that constitute DNA are held together by specific hydrogen bonding between purines and pyrimidines (A pairs with T; and G pairs with C). The size of a nucleic acid molecule is often described in terms of the number of base pairs (symbol: bp) or thousand base pairs (kilobase pairs; symbol: kb; a more convenient unit) it contains.

Binary vector system A two-plasmid system in *Agrobacterium tumefaciens* for transferring into plant cells a segment of T-DNA that carries cloned genes. One plasmid contains the virulence gene (responsible for transfer of the T-DNA), and another plasmid contains the T-DNA borders, the selectable marker and the DNA to be transferred. *See also*cDNA; carrier DNA; plasmid; vector.

Biomass 1. The cell mass produced by a population of living organisms.

2. The organic mass that can be used either as a source of energy or for its chemical components.

3. All the organic matter that derives from the photosynthetic conversion of solar energy.

Biotechnology 1. The use of biological processes or organisms for the production of materials and services of benefit to humankind. Biotechnology includes the use of techniques for the improvement of the characteristics of economically important plants and animals and for the development of micro-organisms to act on the environment.

2. The scientific manipulation of living organisms, especially at the molecular genetic level, to produce new products, such as hormones, vaccines or monoclonal antibodies.

Blot 1. As a verb, this means to transfer DNA, RNA or protein to an immobilizing matrix.

2. As a noun, it usually refers to the autoradiograph produced during the Southern or northern blotting procedures. The variations on this theme depend on the molecules:

– Southern blot: the molecules transferred are DNA molecules, and the probe (q.v.) is DNA.

– northern blot: the molecules transferred are RNA, and the probe is DNA.

– western blot: the molecules transferred are protein, and the probe is labelled antibody.

– Southwestern blot: the molecules transferred are protein, and the probe is DNA.

– dot blot: DNA, RNA or protein are dotted directly onto the membrane support, so that they form discrete spots.

– colony blot: the molecules (usually DNA) are from colonies of bacteria or yeast growing on a bacteriological plate.

cDNA; complementary DNA The double-stranded DNA complement of an mRNA sequence; synthesized in vitro from a mature RNA template using reverse transcriptase (to create a single strand of DNA from the RNA template) and DNA polymerase (to create the double-stranded DNA). Preparation of cDNAs is often the first step in cloning DNA sequences of interest. Used as specific and sensitive probes in hybridization studies, because cDNAs usually do not include regulatory or other controlling sequences, and so they can be used to identify (probe) and isolate genes and their associated sequences from genomic DNA. See binary vector; carrier DNA.

Cloning vector A small, self-replicating DNA molecule - usually a plasmid or viral DNA chromosome - into which foreign DNA is inserted in the process of cloning genes or other DNA sequences of interest. It can carry inserted DNA and be perpetuated in a host cell. Also called a cloning vehicle, vector, or vehicle.

Codon A set of three nucleotides in mRNA, functioning as a unit of genetic coding by specifying a particular amino acid during the synthesis of polypeptides in a cell. A codon specifies a transfer RNA carrying a specific amino acid, which is incorporated into a polypeptide chain during protein synthesis. The specificity for translating genetic information from DNA into mRNA, then to protein, is provided by codon-anticodon pairing. See anticodon; initiation codon; termination codon.

Codon optimization An experimental strategy in which codons within a cloned gene - ones not generally used by the host cell translation system - are changed by in vitro mutagenesis to the preferred codons, without changing the amino acids of the synthesized protein.

Colony 1. An aggregate of identical cells (clones) derived from a single progenitor cell.

2. A group of interdependent cells or organisms.

Constitutive promoter An unregulated promoter that allows for continual transcription of its associated gene.

Cut Slang: to make a double-stranded break in DNA, usually with a type II restriction endonuclease. E.g., "The DNA was cut with *Eco*RI and run out on a 1% agarose gel." *cf* nick; cleave.

Dalton (symbol: Da) A unit of atomic mass roughly equivalent to the mass of a hydrogen atom. 1.67×10^{-24} g. Named after the famous nineteenth-century chemist, John Dalton (1766-1844)). Used in shorthand expressions of molecular weight, especially as kilo- (kDa) or megadaltons (MDa), which are equal to respectively to 1×10^3 and 1×10^6 daltons.

DNA (deoxyribonucleic acid; formerly spelt desoxyribonucleic acid) The long chain of molecules in most cells that carries the genetic message and controls all cellular functions in most forms of life.

DNA ligase An enzyme that catalyses a reaction that links two DNA molecules via the formation of a phospho-diester bond between the 3′ hydroxyl and 5′ phosphate of adjacent nucleotides. It plays an important role in DNA repair and replication. DNA ligase is one of the essential tools of recombinant DNA technology, enabling (among other things) the incorporation of foreign DNA into vectors. The ligase enzyme encoded by phage T4 is commonly used in

gene-cloning experiments. It requires ATP as a co-factor. T4 is used *in vitro* to join the vector and insert DNAs.

DNA polymerase An enzyme that catalyses the synthesis of double-stranded DNA, using single-stranded DNA as a template.

Electrophoresis A technique that separates charged molecules - such as DNA, RNA or protein - on the basis of relative migration in an appropriate matrix (such as agarose gel or polyacrylamide gel) subjected to an electric field. *See* agarose gel electrophoresis; polyacrylamide gel electrophoresis (PAGE); pulsed-field gel electrophoresis (PFGE).

ELISA (enzyme-linked immunosorbent assay) A sensitive technique for accurately determining specific molecules in a mixed sample. The amount of protein or other antigen in a given sample is determined by means of an enzyme-catalysed colour change, avoiding both the hazards and expense of radioactive techniques.

5´ end The phosphate group that is attached to the 5´ carbon atom of a sugar (ribose or deoxyribose) of the terminal nucleotide of a nucleic acid molecule.

Endoplasmic reticulum (Gr. *endon*, within + *plasma*, anything formed or moulded; L. *reticulum*, a small net) A cytoplasmic net of membranes, adjacent to the nucleus, made visible by the electron microscope. Any system of paired membranes within the cytoplasm. Frequently abbreviated to ER. They are sites of protein synthesis.

Enhancer element; enhancer sequence A sequence found in eukaryotes and certain eukaryotic viruses which can increase transcription of a gene when located (in either orientation) up to several kilobases from the gene concerned. These sequences usually act as enhancers when on the 5´ side (upstream) of the gene in question. However, some enhancers are active when placed on the 3´ side (downstream) of the gene. In some cases enhancer elements can activate transcription of a gene with no (known) promoter.

Epitope A specific chemical domain on an antigen that stimulates the production of, and is recognized by, an antibody. Each epitope on a molecule such as protein elicits the synthesis of a different antibody. a.k.a. antigenic determinant.

Epizootic A disease affecting a large number of animals simultaneously.

Gene expression The process by which a gene produces RNA and protein, and hence exerts its effects on the phenotype of an organism.

Immune response The processes, including the synthesis of antibodies, that are used by vertebrates to respond to the presence of a foreign antigen.

Insert A DNA molecule that is incorporated into a cloning vector.

Kanamycin An antibiotic of the aminoglycoside family that poisons translation by binding to the ribosomes.

Ligation The joining of two linear nucleic acid molecules by the formation of phospho-diester bonds. In cloning experiments, a restriction fragment is often ligated to a linearized vector molecule using T4 DNA ligase.

Live vaccine A living, non-virulent form of a micro-organism or virus that is used to elicit an antibody response that will protect the inoculated organism against infection by a virulent form

of the micro-organism or virus. Also a living, non-virulent micro-organism or virus that express a foreign antigenic protein and is used to inoculate humans or animals. The latter organisms are also called a live recombinant vaccines.

Open reading frame (ORF) A sequence of nucleotides in a DNA molecule that has the potential to encode a peptide or protein: it starts with a start triplet (ATG), is followed by a string of triplets each of which encodes an amino acid, and ends with a stop triplet (TAA, TAG or TGA). This term is often used when, after the sequence of a DNA fragment has been determined, the function of the encoded protein is not known. The existence of open reading frames is usually inferred from the DNA (rather than the RNA) sequence.

Promoter 1. A nucleotide sequence of DNA to which RNA polymerase binds and initiates transcription. It usually lies upstream of (5´ to) a coding sequence. A promoter sequence aligns the RNA polymerase so that transcription will initiate at a specific site.

Restriction endonuclease A class of endonucleases that cleaves DNA after recognizing a specific sequence, e.g., BamH1 (5´GGATCC3´), EcoRI (5´GAATTC3´), and HindIII (5´AAGCTT3´). There are three types of restriction endonuclease enzymes:

Type I: Cuts non-specifically a distance greater than 1000 bp from its recognition sequence and contains both restriction and methylation activities.

Type II: Cuts at or near a short, and often palindromic (q.v.) , recognition sequence. A separate enzyme methylates the same recognition sequence. They may make the cuts in the two DNA strands exactly opposite one another and generate blunt ends, or they may make staggered cuts to generate sticky ends. The type II restriction enzymes are the ones commonly exploited in recombinant DNA technology.

Type III: Cuts 24-26 bp downstream from a short, asymmetrical recognition sequence. Requires ATP and contains both restriction and methylation activities.

***Taq* polymerase** A heat-stable DNA polymerase isolated from the thermophilic bacterium *Thermus aquaticus,* and used in PCR.

T-DNA The segment of DNA in the Ti plasmid of Agrobacterium tumefaciens that is transferred to plant cells and inserted into the chromosomes of the plant.

T4 DNA ligase An enzyme from bacteriophage-T4-infected cells, and that catalyses the joining of duplex DNA molecules and repairs nicks in DNA molecules. The enzyme requires that one of the DNA molecules has a 5´-phosphate group and that the other has a free 3´-hydroxyl group.

Terminator (of transcription) A DNA sequence just downstream of the coding segment of a gene, which is recognized by RNA polymerase as a signal to stop synthesizing mRNA. In prokaryotes, terminators usually have an inverted repeat followed by a short stretch of Us at the very end of the transcribed portion. There may also be sequences beyond the transcribed part of the gene which influence the termination of transcription.

Transfection The transfer of DNA to an eukaryotic cell.

Transformation The uptake and establishment of DNA in a bacterium or yeast cell, in which the introduced DNA often changes the phenotype of the recipient organism.

Vaccine A preparation of dead or weakened pathogens, or of derived antigenic determinants, that is used to induce formation of antibodies or immunity against the pathogen.

Vector (L. vehere, to carry) 1. An organism, usually an insect, that carries and transmits disease-causing organisms.

2. A plasmid or phage that is used to deliver selected foreign DNA for cloning and in gene transfer.

Virus (L. *virus*, a poisonous or slimy liquid) An infectious particle composed of a protein capsule and a nucleic acid core (DNA or RNA), which is dependent on a host organism for replication. The DNA or a double-stranded DNA copy of an RNA virus genome is integrated into the host chromosome during lysogenic infection or replicated during the cystic cycle.

11. REFERENCE

Abuodeh, RO, Orbach MJ, Man-del, M.A., Das, A., and Galgiani, J. N. 2000. Genetic transformation of *Coccidioides immitis* facilitated by *Agrobacterium tumefaciens*. J. Infect. Dis. 181, 2106–2110.

Aldous EW, Alexander DJ. 2008. Newcastle disease in pheasants (*Phasianus colchicus*): a review. Vet J. 175:181-5.

Aldous EW, Mynn JK, Banks J. and Alexander DJ. 2003. A molecular epidemiological study of avian paramyxovirus type 1 (Newcastle disease virus) isolates by phylogenetic analysis of a partial nucleotide sequence of the fusion protein gene. Avian Pathology. 32(3): 239-257

Alexander, D.J. 1991. Newcastle Disease and other Paramixovirus Infection. In: Diseases of Poultry, 9th ed. Chap. 19. B.W. Calneak, H.J., Barnes, W.M. Reid, And H.W. Yoder, Jr. Eds. The Iowa State University Press, Ames, Iowa. pp. 496-512.

Alexander, D.J. 2001. Newcastle disease. Br. Poult. Sci., 42:5–22.

Alexander DJ. 2003, Newcastle disease, other avian paramyxoviruses, and pneumovirus infection. In: Disease of poultry, ed.Blackwell, Oxford, UK.

Alexander DJ, J. G. Bell, and R. G. Alders, A Technology Review: Newcastle Disease—with Special Emphasis on Its Effects on Village Chickens, chapter 1–3, Food and Agriculture Organization of the United Nations, Rome, Italy, 2004.

Al-Garib S.O., Gielkens A.L.J., Gruys E. and Koch G. 2003. Review of Newcastle disease virus with particular references to immunity and vaccination. World´s poultry science journal 59:185-200.

Anandalakshmi, R. et al. 1998. A viral suppressor of gene silencing in plants. Proc. Natl. Acad. Sci. U.S.A. 95, 13079–13084

Angulo, E. 2008. Enfermedad de Newcastle Aviar. Vibrac al Dia. Guadalajara Jal. Méx. Realiza: LABORATORIOS VIRBAC MÉXICO S.A. de C.V. 12:1-6

Arzola L, Chen J, Rattanaporn K, Maclean JM and McDonald KA. 2011. Transient Co-Expression of Post-Transcriptional Gene Silencing Suppressors for Increased in Planta

Expression of a Recombinant Anthrax Receptor Fusion Protein. Int. J. Mol. Sci. 12: 4975-4990

Ashby AM, Watson MD, Loake GJ, Shaw CH. Ti plasmid-specified chemotaxis of *Agrobacterium tumefaciens* C58C1 toward vir-inducing phenolic compounds and soluble factors from monocotyledonous and dicotyledonous plants. J Bacteriol 1988; 170:4181-7.

Avesani, L., Marconi, G., Morandini, F., Albertini, E., Bruschetta, M., Bortesi, L., Pezzotti, M., and Porceddu, A. 2007. Stability of Potato virus X expression vectors is related to insert size: implications for replication models and risk assessment, Transgenic Res 16, 587–597.

Aviezer, D., Brill-Almon, E., Shaaltiel, Y., Hashmueli, S., Bartfeld, D., Mizrachi, S., Liberman, Y., Freeman, A., Zimran, A., and Galun, E. 2009. A plant-derived recombinant human glucocerebrosidase enzyme – a preclinical and phase I investigation, PLoS One 4, e4792.

Azhakanandam K, Weissinger SM, Nicholson JS, Qu RD, Weissinger AK. 2007. Amplicon-plus targeting technology (APTT) for rapid production of a highly unstable vaccine protein in tobacco plants. Plant Mol Biol. 63:393–404.

Bakó L, Umeda M, Tiburcio AF, Schell J, Koncz C. 2003. The VirD2 pilot protein of Agrobacterium-transferred DNA interacts with the TATA box-binding protein and a nuclear protein kinase in plants. Proc Natl Acad Sci USA 100: 10108–10113.

Beard, C.W., and R.P. Hanson.: Newcastle Disease. In: Diseases of Poultry. 8th ed., Chapter 19. 2d print. M.S. Hofstad, H.J. Barnes, B.W. Calneak, W.M. Reid, and H.W. Yoder, Jr. eds. The Iowa Satate University Press, Ames Iowa, pp. 452470,1988.

Berinstein A, B. S. Seal, F. Zanetti, A. Kaloghlian, G. Segade, and E. Carrillo. 1999. Newcastle disease virus surveillance in Argentina: use of reverse transcription-polymerase chain reaction and sequencing for molecular typification. Avian Diseases, 43(4): 792–797.

Bevan MW, Flavell RB, Chilton M-D. 1983. A chimeric antibiotic Resistance gene as a selectable marker for plant cell transformation. Nature 304, 184–187.

Bhattacharya A, Sood P, Citovsky V. 2010. The roles of plant phenolics in defence and communication during *Agrobacterium* and *Rhizobium* infection. Mol Plant Pathol; 11:705-19.

Bissonnette, M.L.Z.; Connolly, S.A.; Young, D.F.; Randall, R.E.; Paterson, R.G.; Lamb, R.A. 2006. Analysis of the pH requirement for membrane fusion of different isolates of the paramyxovirus parainfluenza virus 5. J. Virol., 80: 3071–3077.

Botero H. L. A. 2006. Experiencias de Campo en el Manejo y Control de Cepas Altamente Patógenas de la Enfermedad de Newcastle. XI Seminario Internacional de Patología y Producción Aviar.

Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry 72(1-2): 248-254.

Brigneti G. et al. 1998. Viral pathogenicity determinants are suppressors of transgene silencing in Nicotiana benthamiana. EMBO J. 17: 6739–6746

Brosnan CA and Voinnet O. 2009. The long and the short of noncoding RNAs. Curr. Opin. Cell Biol. 21: 416–425

Brown C, King DJ, Seal BS: 1999, Pathogenesis of Newcastle disease in chickens experimentally infected with viruses of different virulence. Vet Pathol 36:125–132.

Bulgakov VP, Kisselev KV, Yakovlev KV, Zhuravlev YN, Gontcharov AA, Odintsova NA. 2006. *Agrobacterium*-mediated transformation of sea urchin embryos. Biotechnol. J. 1, 454–461.

Bundock P, den Dulk-Ras A, Beijersbergen A, Hooykaas PJ. 1995. Trans-kingdom T-DNA transfer from *Agrobacterium tumefaciens* to *Saccharomyces cerevisiae*. EMBO J. 14, 3206–3214.

Burgyán J and Havelda Z. 2011. Viral suppressors of RNA silencing Trends in Plant Science. 16(5):265-272.

Calnek B.W. 2000. Enfermedades de las aves. Segunda Edición. 1110 pp.

California Department of Food and Agriculture. CDFA. Newcastle Disease Information http://www.cdfa.ca.gov/ahfss/Animal_Health/Newcastle_Disease_Info.html Última consulta 12/04/2011.

Cantin C, Holguera J, Ferreira L, Villar E, Munoz-Barroso I. 2007. Newcastle disease virus may enter cells by caveolae-mediated endocytosis. J. Gen. Virol. 88: 559–569.

Carrington J.C. and Freed DD. 1990. Cap-Independent Enhancement of Translation by a Plant Potyvirus 5' Nontranslated Region. JOURNAL OF VIROLOGY. 64(4): 1590-1597.

Cattoli G, Susta L, Terregino C, Brown C. 2011. Newcastle disease: a review of field recognition and current methods of laboratory detection. Journal of Veterinary Diagnostic Investigation 20(10): 1–20

Chambers P, Nesbit M, Yusoff K, Millar NS, Samson AC, Emmerson PT (1988) Location of a neutralizing epitope for the haemagglutinin-neuraminidase glycoprotein of Newcastle disease virus. J. Gen. Virol. 69: 2115–2122.

Chang A and Dutch RE. 2012. Paramyxovirus Fusion and Entry: Multiple Paths to a Common End. Viruses 4: 613-636.

Chen, SL, Lee, W, Hottes, AK, Shapiro, L, McAdams, HH. 2004. Codon usage between genomes is constrained by genome-wide mutational processes. PNAS. 101(10): 3480–3485

Chichester JA and Yusibov V. 2007. Plants as alternative systems for production of vaccines. Hum. Vaccines. 3 (4): 146–148.

Christie PJ, Atmakuri K, Krishnamoorthy V, Jakubowski S, Cascales E. 2005. Biogenesis, architecture, and function of bacterial type IV secretion systems. Annu Rev Microbiol 59: 451–485.

Citovsky V, Kozlovsky SV, Lacroix B, Zaltsman A, Dafny-Yelin M, Vyas S, Tovkach A, Tzfira T. 2007. Biological systems of the host cell involved in Agrobacterium infection. Cellular Microbiology 9(1): 9–20

Cohen J. 1993. Naked DNA points way to vaccines. Science, 259 (5102), 1745–1749.

Conley, A.J., Joensuu, J.J., Menassa, R. and Brandle, J.E. (2009) Induction of protein body formation in plant leaves by elastinlike polypeptide fusions. BMC Biol., 7, 48.

Crennell S, Takimoto T, Portner A, Taylor G. 2000. Crystal structure of the multifunctional paramyxovirus hemagglutinin-neuraminidase. Nat Struct Biol 7:1068–1074.

Czeglédi A, Ujvari D, Somogyia E, Wehmanna E, Werner O, Lomniczi B. 2006. Third genome size category of avian paramyxovirus serotype 1 (Newcastle disease virus) and evolutionary implications. Virus Res. 120:36-48.

Daghir NJ. 2008. Poultry production in hot climates. 2nd Edition. Chapter 1. 1-12.

De Groot MJA, Bundock P, Hooykaas PJJ, Beijersbergen, AGM. 1998. *Agrobacterium tumefaciens*-mediated transformation of filamentous fungi. Nat. Biotechnol. (16): 839–842.

Decker EL and Reski R. 2007. Moss bioreactors producing improved biopharmaceuticals, Curr Opin Biotechnol (18): 393–398.

Decker EL and Reski R. 2008. Current achievements in the production of complex biopharmaceuticals with moss bioreactors, Bioprocess Biosyst Eng (31): 3–9.

De Leeuw OS, Hartog L, Koch G, Peeters BPH. 2003. Effect of fusion protein cleavage site mutations on virulence of Newcastle disease virus: non-virulent cleavage site mutants revert to virulence after one passage in chicken brain. Journal of General Virology. 84(2): 475–484.

De Leeuw OS, Peeters B. 1999. Complete nucleotide sequence of Newcastle disease virus: evidence for the existence of a new genus within the subfamily Paramyxovirinae. J Gen Virol. 80:131-136.

De Lima M, Kwon B, Ansari IH, Pattnaik AK, Flores EF, Osorio FA. 2008. Development of a porcine reproductive and respiratory syndrome virus differentiable (DIVA) strain through deletion of specific immunodominant epitopes. Vaccine. 26: 3594–3600.

Diederich S, Thiel L, Maisner A. 2008. Role of endocytosis and cathepsin-mediated activation in Nipah virus entry. Virology. 375: 391–400.

DiNapoli JM, Ward JM, Cheng L. et al. 2009. Delivery to the lower respiratory tract is required for effective immunization with Newcastle disease virus-vectored vaccines intended for humans. Vaccine. 27(10): 1530–1539.

Ding SW. 2010. RNA-based antiviral immunity. Nat. Rev. Immunol. 10: 632–644

Ding SW and Voinnet O. 2007. Antiviral immunity directed by small RNAs. Cell 130: 413–426

Droual, R., Bickford, A.A., Charlton, B.R. and Kuney, D.R., 1990. Investigation of problems associated with intramuscular breast injection of oil-adjuvanted killed vaccines in chickens, Avian Dis. 34:571–574.

Einhauer A, Jungbauer A. 2001. The FLAG™ peptide, a versatile fusion tag for the purification of recombinant proteins. J. Biochem. Biophys. Methods 49: 455–465

Farkas T, Szekely E, Belak S, Kiss I. 2009. Real-time PCRbased pathotyping of newcastle disease virus by use of TaqMan minor groove binder probes," Journal of Clinical Microbiology, 47(7): 2114–2123.

Farrand SK, van Berkum PB, Oger J. 2003. *Agrobacterium* is a definable genus of the family Rhizobiaceae. Int. J. Syst. Evol. Microbiol. 53: 1681–1687.

Fiedler, U., Phillips, J., Artsaenko, O. and Conrad, U. 1997. Optimization of scFv antibody production in transgenic plants. Immunotechnology, 3: 205–216.

Fischer, R. and Emans, N. 2000. Molecular farming of pharmaceutical proteins. Transgenic Res., 9: 279–299.

Fischer R, Stoger E, Schillberg S, et al. Plant-based production of biopharmaceuticals. Curr Opin Plant Biol 2004; 7: 152-8

Fischer R, Vaquero-Martin C, Sack M, Drossard J, Emans N, Commandeur U. 1999. Towards molecular farming in the future: transient protein expression in plants. Biotechnol. Appl. Biochem. 30 (Pt 2), 113–116.

Flint SJ, Enquist LW, Racaniello VR, Skalka AM. 2007. Attachment and entry, in Principles of Virology, Molecular Biology, Pathogenesis, and Control of Animal Viruses, p. 158, ASM Press,Washington, DC, USA, 2nd edition.

Fraley RT, Rogers SG, Horsch RB, Sanders PR, Flick JS, Adams SP, Bittner ML, Brand LA, Fink CL, Fry JS, Galluppi GR, Goldberg SB, Hoffmann NL, Woo SC. 1983. Expression of bacterial genes in plant cells. Proc.Natl.Acad.Sci.U.S.A. 80: 4803–4807.

Gallili, G. E., and D. Ben-Nathan. 1998. Newcastle disease vaccines. Biotechnol. Adv. 16:343–366.

Gao C, Long D, Lenk I, Nielsen KK (2008) Comparative analysis of transgenic tall fescue (Festuca arundinacea Schreb.) plants obtained by Agrobacterium-mediated transformation and particle bombardment. Plant Cell Rep 27:1601–1609

Gelvin SB. 2003. *Agrobacterium*-mediated plant transformation: the biology behind the 'gene-jockeying' tool. Microbiol Mol Biol Rev. 67: 16–37.

Gelvin SB. 2010. Plant proteins involved in *Agrobacterium*-mediated genetic transformation. Annu Rev Phytopathol. 48:45-68.

Giritch A, Marillonnet S, Engler C, van Eldik G, Botterman J, Klimyuk V, Gleba Y. 2006. Rapid high-yield expression of full-size IgG antibodies in plants coinfected with noncompeting viral vectors. Proc Natl Acad Sci USA. 103:14701–14706.

Gleave A. 1992. A versatile binary vector system with a T-DNA organizational structure conducive to efficient integration of cloned DNA into the plant genome. Plant Molecular Biology 20: 1203-1207.

Gleba Y, Klimyuk V, Marillonnet S. 2005. Magnifection – a new platform for expressing recombinant vaccines in plants. Vaccine. 23: 2042–2048.

Gleba Y, Klimyuk V, Marillonnet S. 2007. Viral vectors for the expression of proteins in plants. Curr Opin Biotechnol. 18: 134–141

Glisson R. J.2006. Interacción de las Enfermedades Respiratorias. XI Seminario Internacional de Patología y Producción Aviar.

Gómez Lim, M. A. 2008. Medicinal Protein Engineering: Capitulo 18, Production of Pharmaceutical Compounds in Plants. CRC Press.

Goodin MM, Zaitlin D, Naidu RA, Lommel SA. 2008. *Nicotiana benthamiana*: its history and future as a model for plant-pathogen interactions, Mol Plant Microbe Interact 21: 1015–1026.

Gravel KA and Morrison TG. 2003. Interacting domains of the HN and F proteins of Newcastle disease virus. J Virol 77:11040–11049.

Grierson, D. y Covey, S. N. 1991. Biologia Molecular de las Plantas. Acriba. Zaragoza, España. 146-198 pp.

Hellwig S, Drossard J, Twyman RM, Fischer R. 2004. Plant cell cultures for the production of recombinant proteins, Nat Biotechnol 22: 1415–1422.

Herczeg J, Wehmann E, Bragg RR, et al. 1999. Two novel genetic groups (VIIb and VIII) responsible for recent Newcastle disease outbreaks in Southern Africa, one (VIIb) of which reached Southern Europe. Arch Virol 144:2087–2099.

Herrera-Estrella L, De Block M, Messens E, Hernalsteens J-P, Van Montagu M, Schell J. 1983. Chimeric genes as dominant selectable markers in plant cells. EMBO J. 2, 987–996.

Hiatt A, Cafferkey R, Bowdish K. 1989. Production of antibodies in transgenic plants, Nature 342: 76–78.

Hiatt A and Pauly M. 2006. Monoclonal antibodies from plants: a new speed record, Proc Natl Acad Sci USA 103: 14645–14646.

Hines NL and Miller CL. 2012. Avian Paramyxovirus Serotype-1: A Review of Disease Distribution, Clinical Symptoms, and Laboratory Diagnostics. Veterinary Medicine International. 1-17.

Huang Z, Chen Q, Hjelm B, Arntzen C, Mason H. 2009. A DNA replicon system for rapid high-level production of viruslike particles in plants, Biotechnol Bioeng 103: 706–714.

Huang, Z., Dry, I., Webster, D., Strugnell, R. and Wesselingh, S. (2001) Plant-derived measles virus hemagglutinin protein induces neutralizing antibodies in mice. Vaccine, 19, 2163–2171.

Iki T. et al. 2010. In vitro assembly of plant RNA-induced silencing complexes facilitated by molecular chaperone HSP90. Mol. Cell 39: 282–291

Iorio RM, Melanson VR, Mahon PJ. 2009. Glycoprotein interactions in paramyxovirus fusion. Future Virol 4:335–351.

Iorio RM, Richard J. Syddall, John P. Sheehan, Michael A. Bratt, Rhona L. Glickman, And Anne M. Riel. 1991. Neutralization Map of the Hemagglutinin-Neuraminidase Glycoprotein of Newcastle Disease Virus: Domains Recognized by Monoclonal Antibodies That Prevent Receptor Recognition. Journal of Virology. 4999-5006

Iyer LM, Kumpatla SP, Chandrasekharan MB, Hall TC (2000) Transgene silencing in monocots. Plant Mol Biol 43:323–346.

James J P, Elizabeth D S, Jeffrey J. 2000. Determination of the disulfide bond arrangement of Newcastle disease virus hemagglutinin neuraminidase. J Biol Chem, 275: 6469–6478

Jauvion V. et al. 2010. The conserved RNA trafficking proteins HPR1 and TEX1 are involved in the production of endogenous and exogenous small interfering RNA in Arabidopsis. Plant Cell 22: 2697–2709

Jennings GT and Bachmann MF. 2008. The coming of age of virus-like particle vaccines. Biol. Chem., 389: 521–536.

Job D. 2002. Plant biotechnology in agriculture. Biochimie 84:1105–1110

Joensuu JJ, Niklander-Teeri V and Brandle JE. 2008. Transgenic plants for animal health: plant-made vaccine antigens for animal infectious disease control. Phytochem Rev 7:553-577

Joh LD, VanderGheynst JS. 2006. Agroinfiltration of plant tissues for production of high-value recombinant proteins: an alternative to production in transgenic crops. J Sci Food Agric. 86: 2002–2004.

Jones, R.C. 2004. Respiratory viral disease–lessons to be learned? International Poultry Producction. 12(4): 11- 13.

Joshi SB, Dutch RE, Lamb RA. 1998. A core trimer of the paramyxovirus fusion protein: Parallels to influenza virus hemagglutinin and HIV-1 gp41. Virology 248:20–34.

Kaiser J. 2008. Is the drought over for pharming? Science 320: 473–475.

Kapczynski, D.R. y King, D.J. (2005). Protection of chickens against overt clinical disease and determination of viral shedding following vaccination with commercially available Newcastle disease virus vaccines upon challenge with highly virulent virus from the California 2002 exotic Newcastle disease outbreak. 2005. *Vaccine* 23 (26), 3424-3433.

Kapila J, DeRycke R, VanMontagu M, Angenon G. 1997. An Agrobacterium-mediated transient gene expression system for intact leaves. Plant Sci.;122:101–108.

Kasschau KD and Carrington JC. 1998. A counterdefensive strategy of plant viruses: suppression of posttranscriptional gene silencing. Cell 95: 461–470

Kersters K and De Ley L. 1984. Genus III. Agrobacterium Conn 1942, 359 AL,"in Bergey's Manual of Systematic Bacteriology, Vol.1, eds N. R. Krieg and J. G. Holt (Baltimore: Williams and Wilkins): 244–254.

Kim LM, D. J. King, D. L. Suarez, C. W. Wong, and C. L. Afonso. 2007. Characterization of class I Newcastle disease virus isolates from Hong Kong live bird markets and detection using real-time reverse transcription-PCR. Journal of Clinical Microbiology, 45(4): 1310–1314.

Kim LM, D. L. Suarez, and C. L. Afonso. 2008. Detection of a broad range of class i and II Newcastle disease viruses using a multiplex real-time reverse transcription polymerase chain reaction assay," Journal of Veterinary Diagnostic Investigation, 20(4): 414–425,.

King, D. J., and B. S. Seal**.** 1997. Biological and molecular characterization of Newcastle disease virus isolates from surveillance of live bird markets in the northeastern United States. Avian Dis. 41**:**683–689.

Knipe DM and P. M. Hetsley. 2001. Paramyxoviridae: the viruses and their replication, in Fields Virology, pp. 1305-1306–1324, Lippincott Williams and Wilkins, Philadelphia, Pa, USA, 4th edition.

Kolokoltsov, A.A.; Deniger, D.; Fleming, E.H.; Roberts, N.J., Jr.; Karpilow, J.M.; Davey, R.A. 2007. Small interfering RNA profiling reveals key role of clathrin-mediated endocytosis and early endosome formation for infection by respiratory syncytial virus. J. Virol. 81: 7786–7800.

Kommers GD, King DJ, Seal BS, Brown CC. 2003. Pathogenesis of chicken-passaged Newcastle disease viruses isolated from chickens and wild and exotic birds. Avian Dis 47:319–329.

Kunik T, Tzfira T, Kapulnik Y, Gafni Y, Dingwall C, Citovsky V. 2001. Genetic transformation of HeLacells by *Agrobacterium*. Proc.Natl.Acad.Sci.U.S.A. 98: 1871–1876.

Kusnadi, A.R., Evangelista, R.L., Hood, E.E., Howard, J.A. and Nikolov, Z.L. 1998. Processing of transgenic corn seed and its effect on the recovery of recombinant betaglucuronidase. Biotechnol. Bioeng. 60, 44–52.

Lacroix B, Tzfira T, Vainstein A, Citovsky V. 2006a. A case of promiscuity: *Agrobacterium'* send-less hunt for new partners. Trends Genet. 22: 29–37.

Lacroix B, Li J, Tzfira T, Citovsky V. 2006b. Will you let me use your nucleus? How *Agrobacterium* gets its T-DNA expressed in the host plant cell. Can. J.Physiol.Pharmacol. 84: 333–345.

Lamb RA and Jardetzky TS. 2007. Structural basis of viral invasion: Lessons from paramyxovirus F. Curr. Opin. Struct. Biol. 17: 427–436.

Lamb RA and Parks GD. 2007. Paramyxoviridae: The viruses and their replication. Fields Virology, eds Knipe DM, Howley PM (Lippincott Williams & Wilkins, Philadelphia), 5th Ed, 1:1449–1496.

Le Bayon JC, Lina B, Rosa-Calatrava M, Boivin G. 2012. Recent developments with live-attenuated recombinant paramyxovirus vaccines. Reviews in Medical Virology.

Leon-Banares R, Gonzalez-Ballester D, Galvan A, Fernandez E. 2004. Transgenic microalgae as green cell-factories, Trends Biotechnol 22: 45–52.

Li J, Krichevsky A, Vaidya M, Tzfira T, Citovsky V. 2005. Uncoupling of the functions of the Arabidopsis VIP1 protein in transient and stable plant genetic transformation by Agrobacterium. Proc Natl Acad Sci USA 102:5733–5738.

Li X, Qiu Y, Yu A, et al. 2009. Degenerate primers based RT-PCR for rapid detection and differentiation of airborne chicken Newcastle disease virus in chicken houses," Journal of Virological Methods. 158(1-2): 1–5.

Lico, C., Chen, Q., and Santi, L. 2008. Viral vectors for production of recombinant proteins in plants, J Cell Physiol 216: 366–377.

Liu H, Y. Zhao, D. Zheng et al. 2011. Multiplex RT-PCR for rapid detection and differentiation of class I and class II Newcastle disease viruses. Journal of Virological Methods. 171(1): pp. 149–155

Liu X, X.Wang, S.Wu et al. 2009. Surveillance for avirulent Newcastle disease viruses in domestic ducks (Anas platyrhynchos and Cairina moschata) at live bird markets in Eastern China and characterization of the viruses isolated," Avian Pathology. 38(5):377–391.

Liu X-S, Yong-lu Wang, Yong-guang Zhang, Yu-zhen Fang, Li Pan, Jian-liang LÜ, Peng Zhou, Zhong-wang Zhang, Shou-tian Jiang. 2011. Analysis of codon usage in peste des petits ruminant's virus. African Journal of Microbiology Research. 5(26): 4592-4600.

Lindbo JA. 2007. TRBO: A High Efficiency Tobacco Mosaic Virus RNA Based Overexpression Vector. Plant Physiol. 145: 1232–1240.

Llave C. 2010. Virus-derived small interfering RNAs at the core of plant–virus interactions. Trends Plant Sci. 15: 701–707

Loyter A, Rosenbluh J, Zakai N, Li J, Kozlovsky SV, Tzfira T, Citovsky V. 2005. The plant VirE2 interacting protein 1. A molecular link between the Agrobacterium T-complex and the host cell chromatin? Plant Physiol 138: 1318–1321.

Ma JK, Drake, P.M. and Christou, P. 2003. The production of recombinant pharmaceutical proteins in plants. Nat. Rev. Genet. 4, 794–805.

Ma P, Liu, J., He, H., Yang, M., Li, M., Zhu, X., and Wang X. 2009. A viral suppressor P1/HC-pro increases the GFP gene expression in agrobacterium-mediated transient assay, Appl Biochem Biotechnol 158: 243–252.

Marillonnet S, Giritch A, Gils M, Kandzia R, Klimyuk V, Gleba Y. 2004. In planta engineering of viral RNA replicons: efficient assembly by recombination of DNA modules delivered by Agrobacterium, Proc Natl Acad Sci USA 101: 6852–6857.

Marillonnet S, Thoeringer C, Kandzia R, Klimyuk V, Gleba Y. 2005. Systemic Agrobacterium tumefaciens-mediated transfection of viral replicons for efficient transient expression in plants, Nat Biotechnol 23: 718–723.

Mas V, Herfst, S.; Osterhaus, A.D.M.E.; Fouchier, R.A.M.; Melero, J.A. 2011. Residues of the human metapneumovirus fusion (F) protein critical for its strain-related fusion phenotype: Implications for the virus replication cycle. J. Virol. 85: 12650–12661.

Matoba N, Magerus A, Geyer BC, Zhang Y, Muralidharan M, Alfsen A, Arntzen CJ, Bomsel M, Mor TS. 2004. A mucosally targeted subunit vaccine candidate eliciting HIV-1 transcytosis-blocking Abs, Proc Natl Acad Sci USA 101: 13584–13589.

Mayo MA. 2002. A summary of taxonomic changes recently approved by ICTV. Arch Virol. 147:1655-1656.

McCormick A. et al. 2008. Plant-produced idiotype vaccines for the treatment of non-Hodgkin'slymphoma: safety and immunogenicity in a phase I clinical study, Proc Natl Acad Sci USA 105: 10131–10136.

McCullen CA and Binns AN. 2006. Agrobacterium tumefaciens and plant cell interactions and activities required for interkingdom macromolecular transfer. Annu Rev Cell Dev Biol 22: 101–127.

Melanson VR and Iorio RM. 2004. Amino acid substitutions in the F-specific domain in the stalk of the newcastle disease virus HN protein modulate fusion and interfere with its interaction with the F protein. J Virol 78:13053–13061.

Melanson VR and Iorio RM. 2006. Addition of N-glycans in the stalk of the Newcastle disease virus HN protein blocks its interaction with the F protein and prevents fusion. J Virol 80:623–633.

Menassa R, Du C, Yin ZQ et al. 2007. Therapeutic effectiveness of orally administered transgenic low-alkaloid tobacco expressing human interleukin-10 in a mouse model of colitis. Plant Biotechnology Journal 5: 50-59

Menassa, R., Nguyen, V., Jevnikar, A. and Brandle, J. (2001) A self-contained system for the field production of plant recombinant interleukin-10. Mol. Breed., 8, 177–185.

Michielse CB, Hooykaas PJJ, van den Hondel CAMJJ, Ram AFJ. 2008. *Agrobacterium*-mediated transformation of the filamentous fungus *Aspergillus awamori*. NatProtoc 3: 1671–1678.

Miller PJ, Decanini EL, Afonso CL. 2010. Newcastle disease: evolution of genotypes and the related diagnostic challenges. Infect Genet Evol 10:26–35.

Miller PJ, Mia Kim L, Ip HS, Afonso CL. 2009. Evolutionary dynamics of Newcastle disease virus. Virology 391: 64–72

Mlotshwa S. et al. 2008. Small RNAs in viral infection and host defense. Trends Plant Sci. 13: 375–382

Moffat, A.S. (1995) Medical applications – exploring transgenic plants as a new vaccine source. Science, 268, 658.

Moloney MM, Holbrook LA. 1997. Subcellular targeting and purification of recombinant proteins in plant production systems. Biotechnology and genetic engineering reviews. 14: 321-336

Morrison TG, 2003. Structure and function of a paramyxovirus fusion protein. Biochimica et Biophysica Acta, 1614(1): 73–84.

Musiychuk K, Natalie Stephenson, Hong Bi, Christine E. Farrance, Goran Orozovic, Maria Brodelius, Peter Brodelius, April Horsey, Natalia Ugulava, Abdel-Moneim Shamloul, Vadim Mett, Shailaja Rabindran, Stephen J. Streatfield, Vidadi Yusibov. 2007. A launch vector for the production of vaccine antigensin plants. Influenza 1: 19–25.

"Newcastle disease," in Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (Mammals, Birds and Bees), chapter 2.3.14, pp. 576–589, World Organization for Animal Health (Office International des Epizooties, OIE), 6th edition, 2008.

O'Keefe BR. et al. 2009. Scaleable manufacture of HIV-1 entry inhibitor griffithsin and validation of its safety and efficacy as a topical microbicide component, Proc Natl Acad Sci USA 106(15):6099–6104.

Obregon, P., Chargelegue, D., Drake, P. M. W., Prada, A., Nuttall, J., Frigerio, L. and Ma, J. K-C. 2006. HIV-1 p24–immunoglobulin fusion molecule: a new strategy for plant-based protein production. *Plant Biotechnology Journal.* 4**:** 195–207

Office International des Epizooties, OIE. 2009. Terrestrial manual. Chapter

Office International des Epizooties, OIE. 2010. Terrestrial manual: THE APPLICATION OF BIOTECHNOLOGY TO THE DEVELOPMENT OF VETERINARY VACCINES. Chapter 1.1.7A.

Orzaez D, Mirabel S, Wieland WH, Granell A. 2006. Agroinjection of tomato fruits. A tool for rapid functional analysis of transgenes directly in fruit. Plant Physiol. 140:3–11.

Paul M and Ma JK-C. 2011. Plant-made pharmaceuticals: Leading products and production platforms. Biotechnology and Applied Biochemistry. 58(1):58-67

Pernet O, Pohl, C.; Ainouze, M.; Kweder, H.; Buckland, R. 2009. Nipah virus entry can occur by macropinocytosis. Virology 395: 298–311.

Perozo F, R. Merino, C. L. Afonso, P. Villegas, and N. Calderon. 2008b. Biological and Phylogenetic Characterization of Virulent Newcastle Disease Virus Circulating in Mexico. AVIAN DISEASES 52:472–479.

Perozo F, P. Villegas, C. Estevez, I. R. Alvarado, L. B. Purvis, and E. Saume. 2008a. Avian Adeno-Associated Virus-Based Expression of Newcastle Disease Virus Hemagglutinin-Neuraminidase Protein for Poultry Vaccination. AVIAN DISEASES 52:253–259.

Phillips JR. et al. 2007. The role of small RNAs in abiotic stress. FEBS Lett. 581: 3592–3597

Piers KL, Heath JD, Liang X, Stephens KM, Nester EW. 1996. *Agrobacterium tumefaciens* mediated transformation of yeast. Proc.Natl.Acad.Sci.U.S.A. 93: 1613–1618.

Plasson C, Michel, R., Lienard, D., Saint-Jore-Dupas, C., Sourrouille, C., de March, GG, Gomord V. 2009. Production of recombinant proteins in suspension-cultured plant cells, Methods Mol Biol 483: 145–161.

Plesha MA, Huang TK, Dandekar AM, Falk BW, McDonald KA. 2007. High-level transient production of a heterologous protein in plants by optimizing induction of a chemically inducible viral amplicon expression system. Biotechnol Prog. 23:1277– 1285.

Plesha MA, Huang TK, Dandekar AM, Falk BW, McDonald KA. 2009. Optimization of the Bioprocessing Conditions for Scale-Up of Transient Production of a Heterologous Protein in Plants Using a Chemically Inducible. Biotechnol. Prog. 25(3):722-734

Pogue GP, Lindbo, J. A., Garger, SJ, Fitzmaurice, WP. 2002. Making an ally from an enemy: plant virology and the new agriculture, Annu Rev Phytopathol. 40: 45–74.

Qu, F., Ren, T., Morris, T.J., 2003. The coat protein of turnip crinkle virus suppresses posttranscriptional gene silencing at an early initiation step. J. Virol. 77, 511–522.

Rakoczy-Trojanowska M (2002) Alternative methods of plant transformation—a short review. Cell Mol Biol Lett 7:849–858

Rao SS, Styles D., Kong W., Andrews C., Gorres J.P. and Nabel GJ. 2009. A gene-based avian influenza vaccine in poultry. Poult. Sci., 88: 860–866.

Rasmusson BJ, Flanagan, T.D.; Turco, S.J.; Epand, R.M.; Petersen, N.O. 1998. Fusion of Sendai virus and individual host cells and inhibition of fusion by lipophosphoglycan measured with image correlation spectroscopy. Biochim. Biophys. Acta Mol. Cell Res.1404: 338–352.

Regensburg-Tuink AJ and Hooykaas PJJ. 1993. Transgenic *N. glauca* plants expressing bacterial virulence gene virF are converted into hosts for nopaline strains of *A. tumefaciens*. Nature 363: 69–71.

Rhodes GH, Abai A.M., Margalith M., Kuwahara-Rundell A., Morrow J., Parker S.E. and Dwarki VJ. 1994. Characterization of humoral immunity after DNA injection. Dev. Biol. Stand., 82: 229–236.

Roth BM, Pruss GJ, Vance VB. 2004. Plant viral suppressors of RNA silencing. Virus Research. 102: 97–108

Ruiz-Ferrer V and Voinnet O. 2009. Roles of plant small RNAs in biotic stress responses. Annu. Rev. Plant Biol. 60: 485–510

Russell CJ and Luque LE. 2006. The structural basis of paramyxovirus invasion. Trends Microbiol 14:243–246.

Rybicki, E.P. 2010. Plant-made vaccines for humans and animals. Plant Biotechnology Journal. 8: 620–637

Ramirez, N., Ayala, M., Lorenzo, D., Palenzuela, D., Herrera, L., Doreste, V., Perez, M., Gavilondo, J.V. and Oramas, P. (2002) Expression of a single-chain Fv antibody fragment specific for the hepatitis B surface antigen in transgenic tobacco plants. Transgenic Res., 11, 61–64.

Sagrera A, Cobaleda C, Gonzalez De Buitrago J M, et al. Membrane glycoproteins of Newcastle disease virus: Nucleotide sequence of the hemagglutinin-neuraminidase cloned gene and structure function relationship of predicted amino acid sequence. Glycoconj J, 2001, 18: 283–289

Saif YM, Fadly AM, Glisson JR, McDougald LR. 2008. Newcastle disease, other avian paramyxoviruses, and pneumovirus infections. In Diseases of Poultry, pp. 75–93, Blackwell Publishing Professional, Ames, Iowa, USA, 12th edition.

Sainsbury F, Liu L, Lomonossoff GP. 2009. Cowpea mosaic virus-based systems for the expression of antigens and antibodies in plants, Methods Mol Biol 483: 25–39.

San Román K, Villar E, Muñoz-Barroso I. 1999. Acidic pH enhancement of the fusion of Newcastle disease virus with cultured cells. Virology. 260: 329–341.

Schob H, Kunc C, Meins F. 1997. Silencing of transgenes introduced into leaves by agroinfiltration: a simple, rapid method for investigating sequence requirements for gene silencing. Mol Gen Genet.256:581–585.

Seal BS. 2004. Nucleotide and predicted amino acid sequence analysis of the fusion protein and hemagglutinin-neuraminidase protein genes among Newcastle disease virus isolates. Phylogenetic relationships among the Paramyxovirinae based on attachment glycoprotein sequences. Funct Integr Genomics 4: 246–257

Seal BS, King DJ, Sellers HS. 2000. The avian response to Newcastle disease virus," Developmental and Comparative Immunology. 24(2-3): 257–268.

Seal BS, M. G. Wise, J. C. Pedersen et al. 2005. Genomic sequences of low-virulence avian paramyxovirus-1 (Newcastle disease virus) isolates obtained from live-bird markets in North America not related to commonly utilized commercial vaccine strains. Veterinary Microbiology, 106(1-2): 7–16

Secretaria de Agricultura, Ganaderia, Desarrollo Rural, Pesca y Alimentacion, SAGARPA. 2012. Registro de Productos Biologicos. http://www.senasica.gob.mx/?id=1484 Última consulta 20/06/2012.

Shaaltiel Y. et al. 2007. Production of glucocerebrosidase with terminal mannose glycans for enzyme replacement therapy of Gaucher's disease using a plant cell system, Plant Biotechnol J 5: 579–590.

Sharp, PM and Li, W-H. 1987. "The codon adaptation index-a measure of directional synonymous codon usage bias, and its potential applications." Nucleic Acids Research 15(3): 1281-1295.

Sheng J and Citovsky V. 1996. Agrobacterium–plant cell interaction: have virulence proteins – will travel. Plant Cell 8: 1699–1710.

Shimoda N, Toyoda-Yamamoto A, Nagamine J, Usami S, Katayama M, Sakagami Y, et al. 1990. Control of expression of Agrobacterium vir genes by synergistic actions of phenolic signal molecules and monosaccharides. Proc Nat Acad Sci USA. 87:6684-8.

Simmons CW, Vandergheynst JS, Upadhyaya SK. 2009. A model of *A. tumefaciens* vacuum infiltration into harvested leaf tissue and subsequent in planta transgene transient expression. Biotechnol Bioeng.;102:965–970.

Smith EC, Popa A, Chang A, Masante C, Dutch RE. 2009. Viral entry mechanisms: The increasing diversity of paramyxovirus entry. FEBS J 276:7217–7227.

Smith ML. et al. 2006. Modified tobacco mosaic virus particles as scaffolds for display of protein antigens for vaccine applications, Virology 348: 475–488.

Smith ML, Fitzmaurice WP, Turpen TH, Palmer KE. 2009. Display of peptides on the surface of tobacco mosaic virus particles, Curr Top Microbiol Immunol 332: 13–31.

Sparrow PA and Twyman RM. 2009. Biosafety, risk assessment and regulation of plant-made pharmaceuticals, Methods Mol Biol 483: 341–353.

Srinivasakumar N, Ogra PL, Flanagan TD. 1991. Characteristics of fusion of respiratory syncytial virus with HEp-2 cells as measured by R18 fluorescence dequenching assay. J. Virol., 65: 4063–4069.

Stanley M. 2008. HPV vaccines: are they the answer? Br. Med. Bull., 88 (1): 59–74.

Stewart, CN Jr. 2008. Plant Biotechnology And Genetics: Principles, Techniques, and Applications. John Wiley & Sons, Inc., Hoboken, New Jersey. Chapter 10. 245-254 pp.

Stewart L. 2007. Gene Synthesis for Protein Production. ENCYCLOPEDIA OF LIFE SCIENCES. 1-12.

Stewart L and Burgin AB (2005) Whole gene synthesis: a gene-o-matic future. In: Atta-ur-Rahman B, Springer A and Caldwell GW (eds) Frontiers in Drug Design and Discovery, pp. 297–341. San Francisco, CA: Bentham Science Publishers.

Stone-Hulslander J and Morrison TG. 1999. Mutational analysis of heptad repeats in the membrane-proximal region of Newcastle disease virus HN protein. J Virol 73:3630–3637.

Sudarshana, M. R., Plesha, M. A., Uratsu, S. L., Falk B. W., Dandekar A. M., Huang, T. K. and McDonald, K. A. 2006. A chemically inducible cucumber mosaic virus amplicon system for expression of heterologous proteins in plant tissues. Plant Biotechnology Journal. 4:551–559

Takeda, A., Sugiyama, K., Nagano, H., Mori, M., Kaido, M., Mise, K., Tsuda, S., Okuno, T., 2002. Identification of a novel RNA silencing suppressor, NSs protein of Tomato spotted wilt virus. FEBS Lett. 532, 75–79.

Takimoto T, Taylor GL, Crennell SJ, Scroggs RA, Portner A. 2000. Crystallization of Newcastle disease virus hemagglutinin-neuraminidase glycoprotein. Virology 270:208–214.

Thanavala Y, Huang Z, Mason HS. 2006. Plant-derived vaccines: a look back at the highlights and a view to the challenges on the road ahead. Expert Rev Vaccines. 5: 249-260

The Center for Food Security and Public Hetlt, CFSPH. Iowa State University. 2008. http://www.cfsph.iastate.edu/Factsheets/es/enfermedad_de_newcastle.pdf Última consulta 12/04/2011.

Toyoda T, Gotoh B, Sakaguchi T, Kida H, Nagai Y (1988) Identification of amino acids relevant to three antigenic determinants on the fusion protein of Newcastle disease virus that are involved in fusion inhibition and neutralization. J. Virol. 62: 4427–4430.

Travella S, Ross SM, Harden J, Everett C, Snape JW, Harwood WA (2005) A comparison of transgenic barley lines produced by particle bombardment and Agrobacterium-mediated techniques. Plant Cell Rep 23:780–789.

Turner R and Foster GD. 1995. The Potential Exploitation of Plant Viral Translational Enhancers in Biotechnology for Increased Gene Expression. Molecular Biotechnology. 3: 225-236.

Tzfira T, Li J, Lacroix B, Citovsky V. 2004. Agrobacterium T-DNA integration: molecules and models. Trends Genet 20: 375–383.

Tzfira T, Vaidya M, Citovsky V. 2001. VIP1, an Arabidopsis protein that interacts with Agrobacterium VirE2, is involved in VirE2 nuclear import and Agrobacterium infectivity. EMBO J. 20:3596-607.

Unión Nacional de Avicultores, México, UNA. http://www.una.org.mx Última consulta 20/06/2012.

Ulmer J.B., Donnelly J.J., Parker S.E., Rhodes G.H., Felgner P.L., Dwarki V.J., Gromkowski S.H., Deck R.R., Dewitt C.M., Friedman A., Et Al. (1993). Heterologous protection against influenza by injection of DNA encoding a viral protein. Science, 259 (5102), 1691–1692.

United States Department of Agriculture, USDA. 2011. Veterinary Biological Products Licensees and Permittees. http://www.aphis.usda.gov Última consulta 20/06/2012.

Vancanneyt, G., Dubald, M., Schroder, W., Peters, J., and Botterman, J. 2009. A case study for plant-made pharmaceuticals comparing different plant expression and production systems, Methods Mol Biol 483, 209–221.

Vaucheret, H. 2006. Post-transcriptional small RNA pathways in plants: mechanisms and regulations. Genes Dev. 20, 759–771

Vergunst, A.C., Schrammeijer, B., den Dulk-Ras, A., de Vlaam, C.M.T., Regensburg-Tuink, T.J., and Hooykaas, P.J.J. 2000. VirB/D4-dependent protein translocation from Agrobacterium into plant cells. Science 290: 979–982.

Vézina, L. P., Faye, L., Lerouge, P., D'Aoust, M. A., Marquet-Blouin, E., Burel, C., Lavoie, P. O., Bardor, M., and Gomord, V. (2009) Transient co-expression for fast and high-yield production of antibodies with human-like N-glycans in plants, Plant Biotechnol J 7, 442–455.

Villani, M. E., Morgun, B., Brunetti, P., Marusic, C., Lombardi, R., Pisoni, I., Bacci, C., Desiderio, A., Benvenuto, E., and Donini, M. (2009) Plant pharming of a full-sized, tumourtargetingantibody using different expression strategies, Plant Biotechnol J 7, 59–72.

Voinnet O. 2009. Origin, biogenesis, and activity of plant microRNAs. Cell 136, 669–687

Voinnet, O., Rivas, S., Mestre, P., Baulcombe, D., 2003. An enhanced transient expression system in plants based on suppression of gene silencing by the p19 protein of tomato bushy stunt virus. Plant J. 33, 949–956.

Wagner, B., Fuchs, H., Adhami, F., Ma, Y., Scheiner, O., and Breiteneder, H. (2004) Plant virus expression systems for transient production of recombinant allergens in Nicotiana benthamiana, Methods 32, 227–234.

Wakamatsu N, King DJ, Kapczynski DR, et al 2006, Experimental pathogenesis for chickens, turkeys, and pigeons of exotic Newcastle disease virus from an outbreak in California during 2002–2003. Vet Pathol 43:925–933.

Wandelt, C.I., Khan, M.R., Craig, S., Schroeder, H.E., Spencer, D. and Higgins, T.J. (1992) Vicilin with carboxy-terminal KDEL is retained in the endoplasmic reticulum and accumulates to high levels in the leaves of transgenic plants. Plant J., 2, 181–192.

Werner S, Breus O, Symonenko Y, Marillonnet S, and Gleba Y. 2011. High-level recombinant protein expression in transgenic plants by using a double-inducible viral vector. PNAS. 108(34): 14061–14066.

White JM, Delos SE, Brecher M, Schornberg K (2008) Structures and mechanisms of viral membrane fusion proteins: Multiple variations on a common theme. Crit Rev Biochem Mol Biol 43:189–219.

Widman D.G., Frolov I. and Mason P.W. (2008). Third-generation flavivirus vaccines based on single-cycle, encapsidation-defective viruses. Adv. Virus Res., 72, 77–126.

Wilczynski SP, Cook ML, Stevens JG. 1977, Newcastle disease as a model for paramyxovirus-induced neurologic syndromes. II: Detailed characterization of the encephalitis. Am J Pathol 89:649–666.

Woodlief WG, Chaplin JF, Campbell CR et al. 1981. Effect of variety and harvest treatments on protein yield of close-grown tobacco. Tob Sci 25:83-86

Wright, F. 1990. "The 'effective number of codons' used in a gene." Gene 87(1): 23-29.

Wroblewski, T., Tomczak, A., and Michelmore,R. (2005) Optimization of Agrobacteriummediated transient assays of gene expression in lettuce, tomato and Arabidopsis, Plant Biotechnol J 3, 259–273.

Wydro, M., Kozubek, E., and Lehmann, P. (2006) Optimization of transient Agrobacterium-mediated gene expression system in leaves of Nicotiana benthamiana, Acta Biochim Pol 53, 289–298.

Xiang C, Han P, Lutziger I, Wang K, Oliver DJ (1999) A mini binary vector series for plant transformation. Plant Mol Biol 40: 711–717

Yamanaka, M., Okabe, T., Nakai, M. and Goto, N., 1993. Local pathological reactions and immune response of chickens to ISA-70 and other adjuvants containing Newcastle disease virus antigen, Avian Dis., 37, 459–466.

Yuan P. et al. 2005. Structural studies of the parainfluenza virus 5 hemagglutininneuraminidase tetramer in complex with its receptor, sialyllactose. Structure 13:803–815.

Yusibov V, Streatfield SJ, Kushnir N. 2011. Clinical development of plantproduced recombinant pharmaceuticals: vaccines, antibodies and beyond. Hum Vaccin 7: 313-321.

Zaltsman A, Krichevsky A, Loyter A, Citovsky V. Agrobacterium induces expression of a plant hostF-box protein required for tumorigenicity. Cell Host Microbe 2010; 7:197-209.

Zanetti F, Berinstein A, Carrillo E. 2008. Effect of host selective pressure on Newcastle disease virus virulence," Microbial Pathogenesis, vol. 44, no. 2, pp. 135–140,.

Zeenko V. and Gallie D.R. 2005. Cap-independent Translation of Tobacco Etch Virus Is Conferred by an RNA Pseudoknot in the 5'-Leader. THE JOURNAL OF BIOLOGICAL CHEMISTRY. 280(29): 26813–26824.

Zhao H and Peeters BPH. 2003. Recombinant Newcastle disease virus as a viral vector: effect of genomic location of foreign gene on gene expression and virus replication. Journal of General Virology. 84(4): 781–788.

Zuckerman JN. (2006). Vaccination against hepatitis A and B: developments, deployment and delusions. Curr. Opin. Infect. Dis., 19 (5), 456–459.

ANNEXES

ANNEX A

Protocol 1. Digestion reaction of 20µl. On an ice bucket, add all the reagents in a 0.6ml centrifuge tube, in this order:

i.	ddWater:	Adjust to final volume of 20 µl
ii.	10x Reaction buffer	2 µl
iii.	plasmid DNA	1 µg
iv.	Restriction enzyme 1	1 µl
v.	Restriction Enzyme 2 (when it is required)	1 µl

Tap to mix and briefly centrifuge, and then place the tube to incubate at 37ºC for two hours. All the restriction enzymes used during this work were from New England Biolabs. Note: see reaction conditions for enzymes different to company said before.

Protocol 2. Receipt of a 0.8% agarose gel and electrophoresis conditions.

i. For 40ml of TAE buffer, weight 0.32g of agarose (Sea Kem® LE Agarose cat#50004).
ii. In a 200ml flask, mix the agarose with the 40ml of TAE buffer and stopper.
iii. Then, melt it using a microwave for 2'22'' at middle power.
iv. Carefully, using a towel place the flask under water flowing and swirl it slowly, until the flask's temperature can be bear with the hand.
v. Add 2.5µl of SYBR® Safe DNA gel stain (cat#533102) and mix it, then decant it in the gel mold, collocate the comb to form the wells and let it dry for 5-10min.
vi. Release the comb and fill the electrophoresis chamber with TAE until the gel is well covered.
vii. Prior to load the samples, on a piece of Parafilm® mix each sample with the loading buffer (6x Glycerol & bromophenol blue) previously pipette it down on the piece of Parafilm® (the amount of loading buffer depends of its concentration and the amount of the sample).
viii. Finally, load each sample in the wells, caring don't leave air bubbles, and run it at 97mV for 25-30min. To visualize the DNA in the gel (bands) place the gel on a transluminator (AlphaImager™2200) with UV light.

Protocol 3. Ligation reaction of 5µl. On an ice bucket, add 1µl of all the components in a 0.2ml centrifuge tube, in this order:

i. ddWater
ii. 5X Reaction buffer (Invitrogen Cat# 15224-017)
iii. Vector DNA
iv. HNop insert DNA
v. T4 DNA ligase (Invitrogen Cat# 15224-017)

Tap to mix and briefly centrifuge, and then place the tube at 4ºC overnight.

Protocol 4. Colony PCR. This protocol can be made with as colonies as can be possible, however are recommended work with until 10 colonies per reaction. Inside of the fume hood, make the procedures as follow:

i. In a 1.5ml centrifuge tube pipette 20µl of ddWater down.
ii. Take a colony with a yellow tip (make sure only take one colony per tip and don't take fused colonies), put it in the 1.5ml centrifuge tube and suspend it with the 20µl of ddWater.
iii. Vertically place the tube in a plastic rack and boil it for 10min in a bath water.
iv. Carefully, release the tube from the bath water and centrifuge it at 13,000rpm for 5min.
v. Pipette 5µl up of the supernatant and pipette them down in a 0.2ml centrifuge tube, then add the PCR solutions in this order:

	Solution	Amount (µl)
a)	ddWater	14.3
b)	10x PCR Buffer	2.5
c)	Primer 1 (Stock of 3µM)	1.25
d)	Primer 2 (Stock of 3µM)	1.25
e)	dNTPs (Invitrogen cat#18427-013)	0.5
f)	AmpliTaq DNA Polymerase (Applied BioSystems cat#N8080152)	0.2

Note: When more than one PCR reaction will be carry out, make a Master Mix (e.g. 10x M.M.) with a 5% of excess of each solution and add 20µl of the M.M. to the 5µl of supernatant.

vi. Tap to mix and briefly centrifuge. Place the tube in the term cycler (GeneAmp PCR system 9700) and set it up with the following conditions:

Cycles #	Temperature (ºC)	Time (min)
1	94	1
30	94	1
	58	1
	68	2
1	4	∞

vii. Once the PCR is finished, load 5µl of PCR product with 2 µl of loading buffer in a 0.8% agarose gel (Protocol 2) and run it in an electrophoresis chamber, with enough TAE buffer to cover the gel, at 97mV for 25-30min. Then, visualize it in a transluminator.

Note: The expected band size is ~1kb using HNop primers with any another primers (TRBO-1, CaMV35S-2 or pQAN-F).

Protocol 5. Dephosphorylation Reaction. If is possible use plasmid DNA fresh.

i. Digest the plasmid DNA in 20µl system (Protocol 1)
ii. Heat it at 75ºC for 10min in a bath water.
iii. Dilute and store on ice Calf Intestinal Alkaline Phosphatase (CIAP, Promega cat#M1821) to 0.01 U/µl as follow:
    a. ddWater 44.5µl
    b. 10x CIAP Buffer 5 µl
    c. CIAP (1 U/µl) 0.5µl

iv. Mix the digested plasmid DNA with the diluted CIAP as follow:

    a. Digested plasmid DNA 17µl
    b. 10x CIAP Buffer 2µl
    c. Diluted CIAP (0.01 U/µl) 1µl

v. Incubate the solution at 37ºC for 30min.
vi. After that, add additional 1µl of diluted CIAP and incubate it as in step five.
vii. Finally, heat deactivate at 75ºC for 10min in a bath water.

Protocol 6. Transformation of *Agrobacterium* by electroporation using a MicroPulser Electroporator (BioRad cat#165-2100).

i. Thaw 40µl aliquot(s) of *Agrobacterium*-competent cells on ice.
ii. Add 1µl of plasmid DNA (2-100ng), mix with the 40µl of cell suspension and let it for 2min on ice.
iii. Transfer 40µl of cells to a pre-cooled 0.2cm electroporation cuvette (BioRad cat#165-2082) and apply the electric pulse immediately. (Push both buttons at the same time until hear a "bip")
iv. Parameters for the electric pulse are following:

    a) Field strength of 2.5kv/cm
    b) Capacitance of 25µfD
    c) Resistors of 400 ohms (200 for *E. coli*) in parallel with the sample
    d) Time constants of 8-12msec (This parameter appears after "the bip" and is to know if the transformation was made correctly)

v. Immediately add 1ml of S.O.C. or similar rich medium and shake it at 28ºC for 45min.
vi. Plated aliquots of 5-15µl on LB 1.5% agar medium containing appropriate antibiotics and incubate for 2-3 days at 28ºC.
vii. Select a colony that had grown well and isolated, culture it in LB medium with appropriate antibiotics at 28ºC overnight with shaking.
viii. After that, make freezer stocks mixing 750µl of cell culture with 250µl of sterile 60% glycerol, and keep it at -80ºC until be used.

Protocol 7. Standard procedure for microtiter plates Bradford Assay (Bio-Rad Protein Assay).

i. Prepare dye reagent by diluting 1 part Dye Reagent Concentrate (cat# 500-0006) with 4 parts ddWater. Filter through a Whatman #1 filter (or equivalent) to remove particulates. This diluted reagent may be used for about 2 weeks when kept at room temperature.

ii. Prepare three to five dilutions of a protein standard, which is representative of the protein solution to be tested. The linear range of this microtiter plate assay is 0.05 mg/ml to approximately 0.5 mg/ml. Protein solutions are normally assayed in duplicate or triplicate.

iii. Pipet 10 µl of each standard and sample solution into separate microtiter plate wells.

iv. Add 200 µl of diluted dye reagent to each well. Mix the sample and reagent thoroughly using a microplate mixer. Alternatively, use a multi-channel pipet to dispense the reagent. Depress the plunger repeatedly to mix the sample and reagent in the well. Replace with clean tips and add reagent to the next set of wells.

v. Incubate at room temperature for at least 5 minutes. Absorbance will increase over time; samples should incubate at room temperature for no more than 1 hour.

vi. Prepare Bradford template in SoftMax software and read at 595nm in SpectraMax 340pc Microplate Reader (Molecular Devices, Sunnyvale, CA)

Protocol 8. NDV-HN protein direct ELISA.

BioReagents and materials

High-binding 96-well plate (Corning Costar 3590, Corning, NY)
5% Non-fat dry milk (5g non-fat dry milk in 100ml 1X PBS, filtered through a Whatman #1 filter)
1X PBS pH 7.4 (NaCl 8g (137 mM); KCl 0.2g (2.7 mM); Na2HPO4 1.44g (10.1 mM); KH2PO4 0.24g (1.8 mM); ddH2O fill to 1000 mL)
1X PBST (PBS + 0.05% Tween 20) pH7.4
Carboxy-terminal FLAG–BAP™ fusion protein (Sigma-Aldrich cat#P7457) as standard.
Mouse mAb ANTI-FLAG® M2 conjugated with horseradish peroxidase (HRP) (Sigma Aldrich cat#A8592)
SureBlue TMB Subtrate (KPL, Gaithersburg, MD)
1N HCl
SpectraMax 340pc Microplate Reader (Molecular Devices, Sunnyvale, CA)

Procedure

Samples and Standard

i. In separate microtubes, dilute samples and controls to an appropriate concentration. The concentration of the samples should be around 1µg/ml. Samples should be assayed in triplicates.
ii. Dilute the standard, C-terminal FLAG–BAP™ fusion protein, **1mg/ml to 5µg/ml (1:200)** using 1X PBS.
iii. In a separate microtube, add 180µl of standard at 5µg/ml. Then add 120µl of 1X PBS to subsequent seven microtubes. Serially dilute the standard by taking 60µl of the previous microtube and mixing into the next microtube. The serial dilution should generate eight standards for the standard curve at the following concentrations (ng/ml): 5,000; 1,666; 555; 185; 61; 20; 7 and 2. Standards should be done in duplicates.
iv. Add 50µl of each sample and standard to the ELISA plate and shake it.
v. Incubate at 37ºC for 45min (Note: The incubation in this step can be longer).
vi. Wash plate four times with 1X PBST and shake plate in between washes.

Blocking

vii. Add to each well 150µl of 5% non-fat dry milk and shake it.
viii. Incubate at 37ºC for 15min.
ix. Wash plate four times with 1X PBST and shake plate in between washes.

Binding of conjugated antibody

x. Dilute conjugated antibody, mouse mAb ANTI-FLAG® M2-HRP, to 1:2000 using 1X PBS.
xi. Add 50µl to each well and shake it.
xii. Incubate at 37ºC for 45min.
xiii. Wash plate four times with 1X PBST and shake plate in between washes.

Detection

xiv. Add 100µl of SureBlue TMB substrate to each well and shake it. (Note: SureBlue TMB reagent must be at room temperature before use).
xv. Incubate at room temperature until see reaction becomes blue (~10min).
xvi. Stop reaction by adding 100µl of 1N HCl to each well and shake it.
xvii. Prepare ELISA template in SoftMax software and read at 450nm in the SpectraMax 340pc Microplate Reader.

Protocol 9. NDV-HN protein detection by Western blot.

BioReagents and materials

5X SDS-PAGE sample buffer C

Tris-HCl (pH 8.2)	312.5 mM
Glycerol	50 % (v/v)
Sodium dodecyl sulfate (SDS)	2 % (v/v)
EDTA	25 mM
Bromophenol blue	0.05 % (wt/v)
Add ddWater to achieve final volume.	

4-15% mini-protean TGX™ gel (Bio Rad cat# 456-1083S)

Prestained standards molecular weight marker (Bio-Rad cat# cat#161-0373)

Thermalcycler or heated block, 95 °C

Mini-PROTEAN 3 electrophoresis cell (Bio Rad)

10X SDS running buffer, Tris/glycine/SDS (BioRad, 161-0732)

1M Dithiothreitol (DTT)

Western Blotting buffer (Store at 4 °C for 24 hr or at -20 °C for 1-2 hr before use)

10X Tris/Glycine buffer (BioRad, 161-0734)	100ml
Methanol	200 mL (20 % v/v)
ddWater	700ml

1X PBS pH 7.4

NaCl	8 g (137 mM)
KCl	0.2 g (2.7 mM)
Na2HPO4	1.44 g (10.1 mM)
KH2PO4	0.24 g (1.8 mM)
ddH2O	Fill to 1000 mL

1X PBST (PBS + 0.05% Tween 20) pH7.4

Sponge (fiber pad)

Filter paper (blotting paper)

0.45 µm nitrocellulose membrane (Bio Rad cat# 162-0091)

Mini Trans-Blot transfer cell (Bio Rad)

Tupperware

5% non-fat dry milk (Safeway) diluted in 1X PBS (NFDM/PBS)

C-terminal FLAG–BAP™ fusion protein (Sigma-Aldrich cat#P7457)

Mouse mAb ANTI-FLAG® M2-horseradish peroxidase (HRP) (Sigma Aldrich cat#A8592)

Procedure

SDS-PAGE

i. Insert 4-15% mini-protean TGX™ gel into Mini-PROTEAN 3 electrophoresis cell following company's directions.
ii. For denature conditions, dilute samples and controls with 5x SDS-PAGE sample buffer C and add 1M DTT at a final concentration of 90 mM. For example: 40µL of sample + 12µL of Sample buffer C + 8µl of 1M DTT.
iii. Heat samples and standard at 95 °C for 5 min using thermacycler / heating block.
iv. Centrifuge for 5 minutes at 12,000 g to spin down any precipitate.
v. Prepare 600 mL of 1X SDS running buffer by diluting 10X BioRad Tris/Glycine/SDS running buffer in ddH2O.
vi. Use 1X SDS running buffer to fill inner chamber and make sure buffer does not leak from inner chamber.
vii. Load 30µl of plant leaf extract samples, controls, standard, and molecular weight marker to each lane.

(Continued protocol 9)

viii. Add remaining 1x SDS running buffer to outside chamber.

ix. Run gel at 200 V for 30 min using power supply.

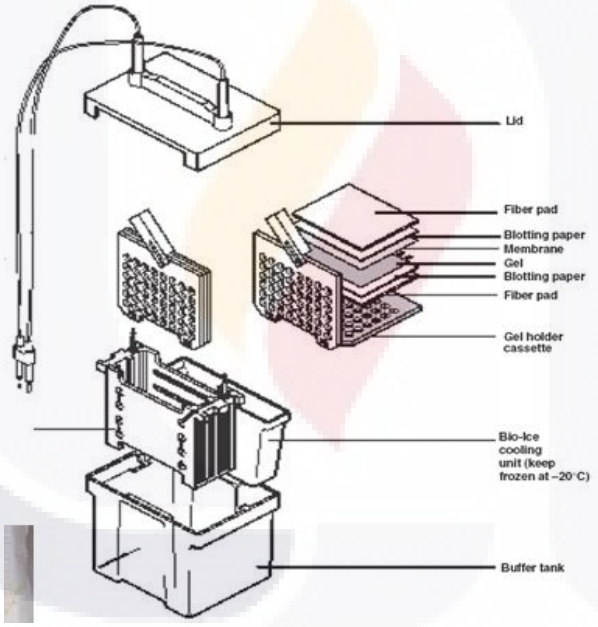
x. Open gel casing and carefully remove gel from cassette ensuring not to rip gel and wash it 3 times in ddWater for 5 min with shacking.

xi. Equilibrate filter papers, gels and 0.45µm nitrocellulose membranes in pre-cooled blotting buffer at 4 °C for 20 min.

xii. Ensure to assemble blot sandwich by starting on the black side of the cassette (the direction of current): Sponge - filter paper - gel - membrane - filter paper - sponge

a. Place soaked sponge on black side of cassette.
b. Place soaked filter paper on sponge.
c. Place soaked gel on filter paper. Remove any air bubbles.
d. Place soaked nitrocellulose membrane on gel. Remove any air bubbles.
e. Place soaked filter paper on membrane. Remove any air bubbles.
f. Place soaked sponge on filter paper.
g. Close cassette, white side on top.

xiii. Place cassette and an ice-cooling unit in Mini Trans-Blot transfer cell (Bio Rad). Add magnetic stir bar to keep consistent temperature throughout the chamber.



xiv. Fill with cold Western blotting buffer and run at 120 V for 80 min to transfer the proteins from the gel to the membrane on a stir plate.

xv. Remove membranes from transfer cell and place in suitable Tupperware and wash them 3 times in 1X PBS for 10min with shaking.

xvi. Block membranes with 5% NFDM/PBS for 15min at room temperature.

xvii. Wash the membranes 3 times in 1X PBST for 5min with shaking.

xviii. Incubate membrane with mouse mAb ANTI-FLAG® M2-HRP diluted at 1:2000 in 5% NFDM/PBS (For example: 10 µL of antibody in 20 ml of 5% NFDM/PBS) at room temperature for 1 hour. And wash as in last step.

xix. Finally, before adding TMB stabilized substrate (Promega cat#W4121), wash membranes with ddWater 3 times x 5min. Color development after 3~15min.

ANNEX B

Table 1. Sequence of native and optimized HN (HNop) constructs and its elements. DNA and amino acid sequence of each element are shown. In addition, all elements are marked with different colors to make easy its location in the sequence.

Sequence of native HN protein excluding signal peptide (HN)
Amino acid (49-571)
EASTPNDLAGISTVISRAEDRVTSLLNSNQDVVDRVYKQVALESPLALLNTESIIMNAITSLSYQINGAANSSGCGAPVHDP DYIGGVGKELIVDDTSDATSFYPSAYQEHLNFIPAPTTGSGCTRIPSFDMSATHYCYTHNVILSGCRDHSHSHQYLALGVL RTSATGRVFFSTLRSINLDDTQNRKSCSVSATPLGCDMLCSKVTETEEEDYKSVTPTSMVHGRLGFDGQYHEKDLDVTV LFKDWVANYPGVGGGSLIDDRVWFPVYGGLKPNSPSDTAQEGKYVIYKRYNNTCPDEQDYQVRMAKSSYKPGRFGGK RVQQAILSIKVSTSLGEDPVLTVPPNTVTLMGAEGRILTVGTSHFLYQRGSSYFSPALLYPMTVRNKTATLHSPYTFNAFT RPGSVPCQASARCPNSCITGVYTDPYPVVFHRNHTLRGVFGTMLDNEQARLNPVSAIFDYTSRSRITRVSSTSTKAAYTT STCFKVVKTNKVYCLSIAEISNTLFGEFRIVPLLVEILKDDRV
cDNA sequence (145-1,716)
GAGGCTAGCACGCCGAACGACCTTGCGGGTATATCGACGGTGATCTCCAGGGCAGAGGATAGGGTTACATCTTTA CTCAATTCAAATCAAGATGTGGTAGATAGGGTATATAAACAGGTGGCCCTTGAGTCCCCGCTGGCGTTGTTGAATA CTGAGTCTATAATTATGAATGCAATAACTTCTCTTTCCTATCAAATTAATGGGGCTGCAAATAGTAGTGGGTGTGGG GCACCTGTTCATGACCCGGATTATATTGGGGGGGTAGGTAAAGAGCTCATAGTAGATGACACGAGTGATGCCACTT CATTCTATCCTTCAGCATATCAAGAACACCTGAACTTTATCCCGGCGCCCACCACAGGTTCAGGCTGCACTCGGAT ACCCTCATTCGACATGAGCGCTACCCACTATTGTTATACTCACAATGTGATATTATCTGGCTGCAGAGATCACTCAC ACTCACATCAGTATTTGGCACTAGGTGTGCTTCGGACATCTGCAACAGGGAGGGTATTCTTTTCTACTCTGCGTTCC ATCAATTTAGATGACACCCAAAATCGGAAGTCTTGCAGTGTGAGTGCAACTCCTTTAGGTTGTGATATGCTGTGCTC TAAAGTCACAGAGACTGAGGAGGAGGATTATAAGTCAGTTACCCCCACATCAATGGTGCATGGAAGGTTAGGGTTT GACGGTCAGTACCATGAGAAGGACTTAGACGTCACAGTCTTATTTAAGGATTGGGTTGCAAATTACCCGGGAGTGG GAGGAGGGTCTCTTATTGACGACCGTGTATGGTTCCCAGTTTATGGAGGGCTAAAACCCAATTCACCTAGCGACAC TGCACAAGAAGGGAAATATGTAATATACAAGCGCTATAATAACACATGCCCCGATGAACAAGATTACCAAGTTCGGA TGGCTAAATCCTCGTATAAGCCTGGACGGTTTGGTGGGAAGCGCGTACAGCAAGCCATCCTATCTATCAAAGTATC AACATCTTTGGGCGAGGACCCGGTGCTGACTGTACCGCCAAATACAGTTACACTCATGGGGGCCGAGGGCAGAAT CCTCACAGTAGGAACATCTCATTTCTTGTACCAGCGAGGGTCTTCATACTTTTCTCCCGCCTTACTATACCCTATGA CAGTGCGCAACAAAACAGCCACTCTTCATAGTCCTTATACATTTAATGCGTTCACTCGGCCGGGTAGTGTCCCTTGC CAGGCATCAGCAAGGTGCCCTAACTCATGTATCACTGGAGTCTATACTGATCCGTACCCTGTAGTCTTCCATAGGA ATCACACCTTGCGAGGGGTGTTCGGGACAATGCTTGATAATGAACAAGCAAGGCTCAATCCCGTATCTGCAATATT TGACTACACATCTCGCAGTCGCATAACCCGGGTAAGTTCGACCAGCACCAAGGCAGCATACACGACATCGACATGT TTTAAAGTTGTCAAGACTAATAAAGTGTATTGTCTTAGCATTGCAGAAATATCCAATACTCTATTTGGGGAATTCAGG ATCGTTCCTTTACTGGTCGAGATTCTCAAAGATGATAGGGTTTAA
Sequence of native HN protein signal peptide (SP-HN)
Amino acid (48)
MDRVVSRVVLENEEREAKNTWRLVFRVAVLSLIVMTLAISVAALVYSM
cDNA (144)
ATGGATCGTGTAGTTAGCAGAGTCGTACTAGAAAACGAAGAAAGAGAAGCAAAGAATACATGGCGCCTGGTTTTCC GGGTCGCAGTCCTATCTCTAATAGTAATGACATTAGCTATCTCTGTAGCCGCCCTGGTATACAGCATG

Sequence of native 2S2 albumin storage protein signal peptide of *Arabidopsis thaliana* (SP-2S2)
Amino acid (21)
MANKLFLVCATFALCFLLTNA
DNA sequence(63)
ATGGCGAACAAACTGTTTCTGGTGTGCGCGACCTTTGCGCTGTGCTTTCTGCTGACCAACGCG
Sequence of native rice α amylase 3D signal peptide (SP-RAmy)
Amino acid (25)
MKNTSSLCLLLLVVLCSLTCNSGQA
DNA sequence (75)
ATGAAGAACACCAGCAGCTTGTGTTTGCTGCTCCTCGTGGTGCTCTGCAGCTTGACCTGTAACTCGGGCCAGGCG
Sequence of native fusion protein 3XFLAG-tag (3XFLAG)
Amino acid (22)
DYKDHDGDYKDHDIDYKDDDDK
DNA sequence (66)
GATTATAAAGATCATGATGGCGATTATAAAGATCATGATATTGATTATAAAGATGATGATGATAAA
Sequence of native endoplasmic reticulum retention signal (KDEL)
Amino acid (4)
KDEL
DNA sequence (12)
AAAGATGAACTG
Sequence of psaDb 5' UTR from *N. sylvestris* (psaDb)
DNA sequence (23)
ACTTCTCTCAATCCAACTTTTCT
Sequence of restriction enzyme sites
5' end
5'spare (**G**) - PacI (**TTAATTAA**) - XhoI (**CTCGAG**) - PstI (**CTGCAG**)
3' end
HindIII (**AAGCTT**) - SpeI (**ACTAGT**) - AvrII (**CCTAGG**) - 3'Spare (**A**)
Sequence of HNop 1 construct
Amino acid (570)
MANKLFLVCATFALCFLLTNADYKDHDGDYKDHDIDYKDDDDKEASTPNDLAGISTVISRAEDRVTSLLNSNQDVVDRVY KQVALESPLALLNTESIIMNAITSLSYQINGAANSSGCGAPVHDPDYIGGVGKELIVDDTSDATSFYPSAYQEHLNFIPAPT TGSGCTRIPSFDMSATHYCYTHNVILSGCRDHSHSHQYLALGVLRTSATGRVFFSTLRSINLDDTQNRKSCSVSATPLGC

DMLCSKVTETEEEDYKSVTPTSMVHGRLGFDGQYHEKDLDVTVLFKDWVANYPGVGGGSLIDDRVWFPVYGGLKPNS
PSDTAQEGKYVIYKRYNNTCPDEQDYQVRMAKSSYKPGRFGGKRVQQAILSIKVSTSLGEDPVLTVPPNTVTLMGAEGR
ILTVGTSHFLYQRGSSYFSPALLYPMTVRNKTATLHSPYTFNAFTRPGSVPCQASARCPNSCITGVYTDPYPVVFHRNHT
LRGVFGTMLDNEQARLNPVSAIFDYTSRSRITRVSSTSTKAAYTTSTCFKVVKTNKVYCLSIAEISNTLFGEFRIVPLLVEIL
KDDRVKDEL

cDNA sequence (1,779)

GTTAATTAACTCGAGCTGCAGACTTCTCTCAATCCAACTTTTCTATGGCGAATAAACTGTTTCTGGTGTGCGCTACC
TTTGCCCTGTGCTTTTTGCTTACCAACGCGGATTACAAAGATCATGATGGAGATTATAAGGATCATGACATTGATTAT
AAAGATGATGATGACAAAGAGGCTAGCACACCGAACGACCTTGCAGGTATATCGACGGTGATCAGTCGCGCTGAG
GACAGGGTTACCTCTTTACTCAATTCCAATCAAGATGTGGTAGATAGGGTATATAAACAGGTTGCCCTTGAAAGCCC
GCTGGCGTTGTTGAATACTGAGTCTATAATTATGAATGCTATAACTTCTCTTTCTTATCAAATTAATGGGGCTGCAAA
TAGTTCCGGATGTGGTGCGCCTGTTCATGATCCTGATTATATAGGGGGAGTAGGTAAGGAGTTAATAGTAGATGAT
ACATCAGATGCCACTTCATTTTACCCGTCAGCATACCAAGAACATCTCAATTTTATCCCAGCGCCTACCACCGGTAG
TGGGTGCACTAGAATACCCTCCTTCGACATGAGCGCTACGCACTATTGTTATACTCATAATGTGATACTATCTGGTT
GCAGAGATCACTCACACAGCCATCAGTATTTGGCTCTAGGTGTGCTTCGGACTTCAGCAACAGGGCGTGTATTCTT
CTCTACTCTGCGTTCCATCAATTTAGATGACACACAAAACCGGAAGTCCTGTAGTGTGTCAGCGACTCCTCTCGGTT
GTGATATGCTATGCTCTAAAGTCACAGAGACTGAGGAAGAGGATTATAAGTCAGTTACCCCAACATCAATGGTGCAT
GGTAGGTTAGGGTTTGACGGTCAGTACCATGAGAAGGATTTGGATGTTACCGTTTTATTCAAAGATTGGGTTGCAAA
TTACCCTGGAGTGGGAGGAGGATCTCTGATTGATGACCGTGTTTGGTTCCCCGTTTATGGTGGGCTAAAACCAAAT
AGTCCAAGCGATACTGCACAAGAAGGCAAATACGTAATTTATAAGCGTTATAATAACACATGCCCTGATGAGCAAGA
TTACCAAGTTAGAATGGCAAAATCCTCGTACAAGCCTGGACGGTTTGGTGGAAAGCGCGTACAGCAAGCAATACTT
TCTATCAAGGTCTCAACATCTTTGGGGGAGGACCCAGTGCTGACTGTACCCCCTAATACAGTGACACTCATGGGGG
CTGAAGGTAGAATCCTCACAGTAGGAACATCTCATTTCTTGTACCAGAGGGGGAGCTCATACTTTTCTCCGGCTTTA
CTATACCCTATGACAGTCAGAAACAAAACAGCAACTCTTCATAGTCCTTATACATTTAATGCGTTCACTCGGCCGGG
TAGTGTCCCTTGCCAGGCGTCAGCAAGGTGCCCTAACTCATGTATTACTGGAGTCTATACTGATCCGTACCCTGTA
GTCTTTCATAGGAATCACACCTTGAGAGGGGTGTTTGGGACAATGCTTGATAATGAACAGGCAAGGCTCAATCCGG
TCAGTGCAATTTTTGACTACACATCTAGAAGTCGCATCACCCGCGTTAGTTCGACCTCTACGAAGGCAGCTTATACG
ACATCGACATGTTTTAAAGTTGTTAAGACTAATAAAGTGTACTGTCTTTCCATTGCAGAAATATCCAATACGCTCTTC
GGGGAATTCAGGATCGTTCCATTACTGGTCGAGATTTTGAAGGATGACAGGGTGAAAGATGAACTGTGATAA**AAGC
TTACTAGTCCTAGGA**

Sequence of HNop 2 construct

Amino acid (597)

MDRVVSRVVLENEEREAKNTWRLVFRVAVLSLIVMTLAISVAALVYSMDYKDHDGDYKDHDIDYKDDDDKEASTPNDLA
GISTVISRAEDRVTSLLNSNQDVVDRVYKQVALESPLALLNTESIIMNAITSLSYQINGAANSSGCGAPVHDPDYIGGVGKE
LIVDDTSDATSFYPSAYQEHLNFIPAPTTGSGCTRIPSFDMSATHYCYTHNVILSGCRDHSHSHQYLALGVLRTSATGRVF
FSTLRSINLDDTQNRKSCSVSATPLGCDMLCSKVTETEEEDYKSVTPTSMVHGRLGFDGQYHEKDLDVTVLFKDWVAN
YPGVGGGSLIDDRVWFPVYGGLKPNSPSDTAQEGKYVIYKRYNNTCPDEQDYQVRMAKSSYKPGRFGGKRVQQAILSI
KVSTSLGEDPVLTVPPNTVTLMGAEGRILTVGTSHFLYQRGSSYFSPALLYPMTVRNKTATLHSPYTFNAFTRPGSVPCQ
ASARCPNSCITGVYTDPYPVVFHRNHTLRGVFGTMLDNEQARLNPVSAIFDYTSRSRITRVSSTSTKAAYTTSTCFKVVK
TNKVYCLSIAEISNTLFGEFRIVPLLVEILKDDRVKDEL

cDNA sequence (1,860)

GTTAATTAACTCGAGCTGCAGACTTCTCTCAATCCAACTTTTCTATGGATAGGGTGGTTTCAAGAGTGGTACTTGAA
AACGAAGAAAGAGAAGCAAAGAACACATGGAGATTGGTGTTCAGGGTGGCAGTCTTAAGTTTAATCGTGATGACCT
TAGCTATTTCTGTAGCCGCCCTGGTGTATAGCATGGATTATAAAGATCACGATGGCGATTATAAGGATCACGATATT
GATTATAAAGACGATGATGATAAAGAGGCTAGCACGCCGAACGACCTCGCGGGTATCAGTACGGTCATATCCAGG
GCAGAAGATAGAGTTACATCCCTTTTAAATTCAAATCAAGATGTTGTAGATAGGGTATATAAACAGGTGGCGTTAGA

GTCCCCGCTTGCGTTGCTTAATACTGAGTCTATAATTATGAATGCTATTACAAGTCTTTCCTATCAGATTAACGGGGC
AGCAAATAGTTCGGGCTGCGGGGCTCCTGTACATGATCCAGATTATATTGGGGGGGTCGGTAAAGAGCTGATTGT
GGACGACACATCCGATGCCACTTCTTTCTACCCTTCAGCATATCAAGAACACCTGAACTTCATTCCTGCTCCAACGA
CCGGTAGTGGATGCACTAGGATCCCTTCATTCGACATGTCTGCTACCCACTATTGCTATACTCACAATGTTATATTAT
CTGGTTGCAGGGACCACTCTCACAGCCATCAGTATCTAGCTTTGGGTGTCCTTCGGACATCTGCAACTGGAAGGGT
TTTTTTTTCCACTTTGCGTTCCATTAACTTAGATGATACTCAAAATAGGAAGAGTTGCAGTGTAAGTGCAACTCCTTT
GGGTTGTGATATGCTGTGCTCTAAGGTAACAGAAACAGAGGAGGAAGATTATAAGTCAGTTACCCCCACATCAATG
GTGCATGGGAGATTAGGCTTTGATGGGCAGTATCACGAGAAAGATTTAGACGTTACAGTTTTATTCAAGGATTGGGT
TGCAAATTACCCGGGTGTGGGCGGAGGGAGCTTAATTGACGACCGTGTATGGTTCCCAGTCTACGGAGGCCTGAA
GCCTAACTCACCTAGCGATACTGCACAAGAAGGGAAATATGTCATTTATAAGCGGTATAACAATACCTGTCCTGATG
AACAGGATTATCAAGTAAGAATGGCTAAATCATCGTATAAGCCTGGACGGTTTGGTGGAAAGCGGGTCCAGCAAGC
CATCCTATCTATCAAAGTTTCAACGTCTTTGGGCGAGGACCCTGTGCTGACTGTGCCGCCTAATACAGTGACATTGA
TGGGGGCTGAGGGCCGGATCCTCACTGTAGGTACGTCTCATTTCCTATATCAGCGGGGTTCTAGTTATTTTTCTCC
CGCCCTGTTGTACCCTATGACAGTGCGCAACAAAACGGCAACTCTTCACAGCCCATATACATTCAATGCATTCACTA
GGCCAGGTTCAGTTCCTTGTCAAGCATCAGCAAGGTGCCCTAACTCTTGTATAACTGGAGTCTATACCGATCCTTAT
CCTGTAGTTTTCCATCGTAATCACACATTGCGAGGGGTATTCGGGACAATGCTTGATAATGAACAAGCAAGGTTGAA
TCCTGTTTCTGCCATATTTGATTACACAAGTCGCTCACGCATAACGAGGGTTAGCTCAACCAGCACCAAAGCAGCAT
ACACAACATCGACATGTTTTAAAGTTGTCAAGACAAATAAAGTGTACTGTCTTAGCATTGCAGAGATTTCTAATACTC
TTTTTGGAGAGTTTCGCATCGTACCTCTTCTAGTTGAGATCCTAAAAGACGATCGTGTTAAGGACGAACTGTGATAA
AAGCTTACTAGTCCTAGGA

Sequence of HNop 3 construct

Amino acid (570)

MKNTSSLCLLLLVVLCSLTCNSGQADYKDHDGDYKDHDIDYKDDDDKEASTPNDLAGISTVISRAEDRVTSLLNSNQDVV
DRVYKQVALESPLALLNTESIIMNAITSLSYQINGAANSSGCGAPVHDPDYIGGVGKELIVDDTSDATSFYPSAYQEHLNFI
PAPTTGSGCTRIPSFDMSATHYCYTHNVILSGCRDHSHSHQYLALGVLRTSATGRVFFSTLRSINLDDTQNRKSCSVSAT
PLGCDMLCSKVTETEEEDYKSVTPTSMVHGRLGFDGQYHEKDLDVTVLFKDWVANYPGVGGGSLIDDRVWFPVYGGL
KPNSPSDTAQEGKYVIYKRYNNTCPDEQDYQVRMAKSSYKPGRFGGKRVQQAILSIKVSTSLGEDPVLTVPPNTVTLMG
AEGRILTVGTSHFLYQRGSSYFSPALLYPMTVRNKTATLHSPYTFNAFTRPGSVPCQASARCPNSCITGVYTDPYPVVFH
RNHTLRGVFGTMLDNEQARLNPVSAIFDYTSRSRITRVSSTSTKAAYTTSTCFKVVKTNKVYCLSIAEISNTLFGEFRIVPL
LVEILKDDRV

cDNA sequence (1,779)

GTTAATTAACTCGAGCTGCAGACTTCTCTCAATCCAACTTTTCTATGAAGAATACAAGTAGCTTGTGTCTCCTATTG
CTCGTTGTCCTCTGTTCACTCACCTGTAATTCCGGCCAAGCCGACTATAAAGATCACGATGGTGACTACAAAGATCA
TGACATCGACTACAAAGACGACGATGATAAGGAGGCATCTACACCAAACGATCTTGCTGGAATCAGTACTGTTATAT
CCAGGGCGGAAGACAGAGTCACAAGCCTTTTAAATTCTAACCAGGACGTCGTGGACAGAGTTTATAAACAAGTTGC
TCTCGAAAGCCCTCTAGCCCTGCTGAATACCGAAAGCATTATAATGAATGCTATTACATCATTGTCGTACCAAATCA
ATGGAGCTGCCAATAGTTCTGGTTGTGGTGCACCGGTGCACGATCCAGATTATATTGGTGGGGTCGGAAAGGAAC
TTATTGTGGATGATACGAGTGACGCGACATCATTTTACCCTTCGGCTTATCAGGAACACCTCAATTTCATCCCTGCT
CCTACTACTGGATCAGGCTGTACTCGGATACCTTCTTTTGATATGTCAGCTACCCATTATTGCTACACCCATAACGT
AATTTTGTCTGGATGCCGTGATCACTCACATAGTCATCAATACCTCGCTTTGGGGGTTTTACGCACCTCCGCGACTG
GACGTGTCTTTTTCAGTACCCTTAGATCTATTAACCTTGATGATACACAAAACCGGAAGTCTTGTTCAGTGTCAGCAA
CACCTTTGGGGTGTGACATGCTGTGCAGCAAGGTTACCGAGACGGAAGAAGAAGATTACAAATCAGTTACACCAAC
CAGCATGGTGCATGGTAGACTTGGTTTTGACGGTCAGTACCATGAAAAGGATTTGGATGTAACAGTTTTGTTTAAGG
ACTGGGTGGCTAACTATCCAGGCGTCGGAGGAGGGAGCCTTATAGATGACCGTGTTTGGTTCCCAGTGTATGGAG
GCCTTAAACCAAACTCCCCTTCTGATACTGCTCAGGAGGGTAAGTATGTAATTTATAAGCGTTATAATAATACATGCC
CTGATGAGCAAGATTATCAGGTTCGTATGGCGAAGTCTTCCTATAAACCAGGAAGATTTGGTGGGAAGCGGGTTCA
GCAAGCAATACTTTCTATAAAGGTCTCCACTAGCCTGGGTGAGGACCCAGTATTGACAGTCCCTCCTAATACAGTG
ACGCTGATGGGGGCCGAAGGTAGAATTCTCACAGTTGGGACTTCTCATTTTCTGTATCAAAGAGGATCATCGTACTT

CTCGCCAGCCTTGTTGTATCCAATGACAGTTAGAAACAAGACTGCAACTTTGCACTCACCATATACTTTCAATGCTTT
TACTAGACCAGGCTCGGTTCCATGTCAGGCATCAGCAAGGTGTCCAAATTCTTGTATAACTGGCGTCTATACAGATC
CTTATCCTGTAGTTTTCCATAGGAATCACACTCTACGAGGCGTGTTCGGAACAATGTTAGATAACGAACAAGCAAGG
CTGAACCCTGTGTCTGCCATTTTTGATTACACAAGCCGCTCCCGAATTACGAGAGTATCTTCTACGTCCACAAAGGC
TGCATATACAACATCAACATGCTTTAAGGTTGTCAAGACAAACAAGGTGTACTGTCTGTCTATTGCCGAAATCTCCA
ATACATTATTCGGGGAGTTTCGTATAGTTCCTCTGCTCGTCGAGATTTTAAAGGATGATCGAGTTTGATAA**AAGCTT**
ACTAGTCCTAGGA

Sequence of HNop 4 construct

Amino acid (597)

MDRVVSRVVLENEEREAKNTWRLVFRVAVLSLIVMTLAISVAALVYSMDYKDHDGDYKDHDIDYKDDDDKEASTPNDLA
GISTVISRAEDRVTSLLNSNQDVVDRVYKQVALESPLALLNTESIIMNAITSLSYQINGAANSSGCGAPVHDPDYIGGVGKE
LIVDDTSDATSFYPSAYQEHLNFIPAPTTGSGCTRIPSFDMSATHYCYTHNVILSGCRDHSHSHQYLALGVLRTSATGRVF
FSTLRSINLDDTQNRKSCSVSATPLGCDMLCSKVTETEEEDYKSVTPTSMVHGRLGFDGQYHEKDLDVTVLFKDWVAN
YPGVGGGSLIDDRVWFPVYGGLKPNSPSDTAQEGKYVIYKRYNNTCPDEQDYQVRMAKSSYKPGRFGGKRVQQAILSI
KVSTSLGEDPVLTVPPNTVTLMGAEGRILTVGTSHFLYQRGSSYFSPALLYPMTVRNKTATLHSPYTFNAFTRPGSVPCQ
ASARCPNSCITGVYTDPYPVVFHRNHTLRGVFGTMLDNEQARLNPVSAIFDYTSRSRITRVSSTSTKAAYTTSTCFKVVK
TNKVYCLSIAEISNTLFGEFRIVPLLVEILKDDRVKDEL

cDNA sequence (1,837)

GTTAATTAACTCGAGCTGCAGATGGATAGGGTGGTTTCAAGAGTGGTACTTGAAAACGAAGAAAGAGAAGCAAAGA
ACACATGGAGATTGGTGTTCAGGGTGGCAGTCTTAAGTTTAATCGTGATGACCTTAGCTATTTCTGTAGCCGCCCTG
GTGTATAGCATGGATTATAAAGATCACGATGGCGATTATAAGGATCACGATATTGATTATAAAGACGATGATGATAA
AGAGGCTAGCACGCCGAACGACCTCGCGGGTATCAGTACGGTCATATCCAGGGCAGAAGATAGAGTTACATCCCT
TTTAAATTCAAATCAAGATGTTGTAGATAGGGTATATAAACAGGTGGCGTTAGAGTCCCCGCTTGCGTTGCTTAATA
CTGAGTCTATAATTATGAATGCTATTACAAGTCTTTCCTATCAGATTAACGGGGCAGCAAATAGTTCGGGCTGCGGG
GCTCCTGTACATGATCCAGATTATATTGGGGGGGTCGGTAAAGAGCTGATTGTGGACGACACATCCGATGCCACTT
CTTTCTACCCTTCAGCATATCAAGAACACCTGAACTTCATTCCTGCTCCAACGACCGGTAGTGGATGCACTAGGATC
CCTTCATTCGACATGTCTGCTACCCACTATTGCTATACTCACAATGTTATATTATCTGGTTGCAGGGACCACTCTCAC
AGCCATCAGTATCTAGCTTTGGGTGTCCTTCGGACATCTGCAACTGGAAGGGTTTTTTTTTCCACTTTGCGTTCCAT
TAACTTAGATGATACTCAAAATAGGAAGAGTTGCAGTGTAAGTGCAACTCCTTTGGGTTGTGATATGCTGTGCTCTA
AGGTAACAGAAACAGAGGAGGAAGATTATAAGTCAGTTACCCCCACATCAATGGTGCATGGGAGATTAGGCTTTGA
TGGGCAGTATCACGAGAAAGATTTAGACGTTACAGTTTTATTCAAGGATTGGGTTGCAAATTACCCGGGTGTGGGC
GGAGGGAGCTTAATTGACGACCGTGTATGGTTCCCAGTCTACGGAGGCCTGAAGCCTAACTCACCTAGCGATACT
GCACAAGAAGGGAAATATGTCATTTATAAGCGGTATAACAATACCTGTCCTGATGAACAGGATTATCAAGTAAGAAT
GGCTAAATCATCGTATAAGCCTGGACGGTTTGGTGGAAAGCGGGTCCAGCAAGCCATCCTATCTATCAAAGTTTCA
ACGTCTTTGGGCGAGGACCCTGTGCTGACTGTGCCGCCTAATACAGTGACATTGATGGGGGCTGAGGGCCGGATC
CTCACTGTAGGTACGTCTCATTTCCTATATCAGCGGGGTTCTAGTTATTTTTCTCCCGCCCTGTTGTACCCTATGAC
AGTGCGCAACAAAACGGCAACTCTTCACAGCCCATATACATTCAATGCATTCACTAGGCCAGGTTCAGTTCCTTGTC
AAGCATCAGCAAGGTGCCCTAACTCTTGTATAACTGGAGTCTATACCGATCCTTATCCTGTAGTTTTCCATCGTAAT
CACACATTGCGAGGGGTATTCGGGACAATGCTTGATAATGAACAAGCAAGGTTGAATCCTGTTTCTGCCATATTTGA
TTACACAAGTCGCTCACGCATAACGAGGGTTAGCTCAACCAGCACCAAAGCAGCATACACAACATCGACATGTTTT
AAAGTTGTCAAGACAAATAAAGTGTACTGTCTTAGCATTGCAGAGATTTCTAATACTCTTTTTGGAGAGTTTCGCATC
GTACCTCTTCTAGTTGAGATCCTAAAAGACGATCGTGTTAAGGACGAACTGTGATAA**AAGCTTACTAGTCCTAGGA**

Analysis 1. Codon Optimization. It was carry out using Gene Design Developer 1.0 (Jung and McDonald 2011). To achieve codon optimization and gene engineering, features such as repeated DNA sequence, potencial polyadenylation signal, potencial polyadenylation signal, and restriction enzyme sites. Each feature is indicated in the HNop constructs as “Original” (Native or non-optimized) and “Optimized” (Codon Optimized). Reduction of undesired sequence are shown in optimized constructs.

HNop1 “Original”: 1713 bp
HNop1 “Optimized”: 1713 bp
Number of mismatched bases: 181 (10,57%)
Number of mismatched codons: 150 (26,27%)

HNop2 “Original”: 1794 bp
HNop2 “Optimized”: 1794
Number of mismatched bases: 268 (14,94%)
Number of mismatched codons: 222 (37,12%)

HNop3 “Original”: 1713 bp
HNop3 “Optimized”: 1713 bp
Number of mismatched bases: 430 (25,10%)
Number of mismatched codons: 361 (63,22%)

HNop4 “Original”: 1794 bp
HNop4 “Optimized”: 1794
Number of mismatched bases: 268 (14,94%)
Number of mismatched codons: 222 (37,12%)

REPEATED DNA SEQUENCE
HNop 1

ORIGINAL

[F] GATTATAAAGAT (12 bp) : 64, 85, 106
[F] ATTATAAAGATC (12 bp) : 65, 86
[F] TTATAAAGATCA (12 bp) : 66, 87
[F] TATAAAGATCAT (12 bp) : 67, 88
[F] ATAAAGATCATG (12 bp) : 68, 89
[F] TAAAGATCATGA (12 bp) : 69, 90
[F] AAAGATCATGAT (12 bp) : 70, 91
[F] GATAGGGTTA (10 bp) : 187, 1690
[F] TAGATGACAC (10 bp) : 410, 671
[B] ATAGGGTATA (10 bp) : 230, 1271
[B] CAGCATATCA (10 bp) : 446, 735
[B] CACTCACACT (10 bp) : 580, 709

OPTIMIZED

[F] GAACATCTCA (10 bp) : 457, 1208
[B] AGGATCATGA (10 bp) : 92, 375
[B] ATAGGGTATA (10 bp) : 230, 1271
[B] GCCTGTTCAT (10 bp) : 360, 1475

[F]:Forward [B]:Backward Complementary
[P]:Palindrome [C]:Connected

HNop 2

ORIGINAL
[F] GATTATAAAGAT (12 bp) : 145, 166, 187
[F] ATTATAAAGATC (12 bp) : 146, 167
[F] TTATAAAGATCA (12 bp) : 147, 168
[F] TATAAAGATCAT (12 bp) : 148, 169
[F] ATAAAGATCATG (12 bp) : 149, 170
[F] TAAAGATCATGA (12 bp) : 150, 171
[F] AAAGATCATGAT (12 bp) : 151, 172
[F] GATAGGGTTA (10 bp) : 268, 1771
[F] TAGATGACAC (10 bp) : 491, 752
[B] GAAAGAGAAG (10 bp) : 40, 398
[B] ATAGGGTATA (10 bp) : 311, 1352
[B] CAGCATATCA (10 bp) : 527, 816
[B] CACTCACACT (10 bp) : 661, 790

OPTIMIZED
[F] GATTATAAAGA (11 bp) : 145, 187
[B] CAGCATATCA (10 bp) : 527, 816
[B] GAAGAGTTGC (10 bp) : 771, 1381

[F]:Forward [B]:Backward Complementary
[P]:Palindrome [C]:Connected

HNop 3

ORIGINAL

[F] GATTATAAAGAT (12 bp) : 76, 97, 118
[F] ATTATAAAGATC (12 bp) : 77, 98
[F] TTATAAAGATCA (12 bp) : 78, 99
[F] TATAAAGATCAT (12 bp) : 79, 100
[F] ATAAAGATCATG (12 bp) : 80, 101
[F] TAAAGATCATGA (12 bp) : 81, 102
[F] AAAGATCATGAT (12 bp) : 82, 103
[F] GATAGGGTTA (10 bp) : 199, 1702
[F] TAGATGACAC (10 bp) : 422, 683
[B] ATAGGGTATA (10 bp) : 242, 1283
[B] CAGCATATCA (10 bp) : 458, 747
[B] CACTCACACT (10 bp) : 592, 721

OPTIMIZED

[F] GACTACAAAGA (11 bp) : 97, 118
[B] GTGCACCGGTGC (12 bp) : 368, 381
[B] TGCACCGGTGCA (12 bp) : 369, 380 [P]
[B] GCACCGGTGCAC (12 bp) : 370, 379
[B] GTTTATAAAC (10 bp) : 247, 256 [P]
[B] CATTATAATG (10 bp) : 300, 309 [P]

[F]:Forward [B]:Backward Complementary
[P]:Palindrome [C]:Connected

HNop 4

ORIGINAL

[F] GATTATAAAGAT (12 bp) : 145, 166, 187
[F] ATTATAAAGATC (12 bp) : 146, 167
[F] TTATAAAGATCA (12 bp) : 147, 168
[F] TATAAAGATCAT (12 bp) : 148, 169
[F] ATAAAGATCATG (12 bp) : 149, 170
[F] TAAAGATCATGA (12 bp) : 150, 171
[F] AAAGATCATGAT (12 bp) : 151, 172
[F] GATAGGGTTA (10 bp) : 268, 1771
[F] TAGATGACAC (10 bp) : 491, 752
[B] GAAAGAGAAG (10 bp) : 40, 398
[B] ATAGGGTATA (10 bp) : 311, 1352
[B] CAGCATATCA (10 bp) : 527, 816
[B] CACTCACACT (10 bp) : 661, 790

OPTIMIZED

[F] GATTATAAAGA (11 bp) : 145, 187
[B] CAGCATATCA (10 bp) : 527, 816
[B] GAAGAGTTGC (10 bp) : 771, 1381

[F]:Forward [B]:Backward Complementary
[P]:Palindrome [C]:Connected

POTENCIAL POLYADENYLATION SIGNAL

HNop 1

ORIGINAL

[F] GTAAGT : 1 finding(s) at 1534
[F] AATAAA : 1 finding(s) at 1597
[F] CATAAA :
[F] GATAAA : 1 finding(s) at 124
[F] TATAAA : 4 finding(s) at 67, 88, 109, 238
[F] ACTAAA :
[F] AGTAAA :
[F] ATTAAA :
[F] AAAAAA :
[F] AACAAA : 2 finding(s) at 7, 1282
[F] AAGAAA :
[F] AATCAA : 1 finding(s) at 214
[F] AATGAA : 1 finding(s) at 1465
[F] AATTAA : 1 finding(s) at 324
[F] AATACA : 1 finding(s) at 1162
[F] AATAGA :
[F] AATATA : 1 finding(s) at 990
[F] AATAAC : 2 finding(s) at 303, 1006
[F] AATAAG :
[F] AATAAT :
[F] AATATT : 1 finding(s) at 1497
[F] AATAAA : 1 finding(s) at 1597
[F] TTTGTA :

[F]:Forward

OPTIMIZED

[F] GTAAGT :
[F] AATAAA : 2 finding(s) at 7, 1597
[F] CATAAA :
[F] GATAAA :
[F] TATAAA : 2 finding(s) at 109, 238
[F] ACTAAA :
[F] AGTAAA :
[F] ATTAAA :
[F] AAAAAA :
[F] AACAAA : 1 finding(s) at 1282
[F] AAGAAA :
[F] AATCAA : 1 finding(s) at 214
[F] AATGAA : 1 finding(s) at 1465
[F] AATTAA : 1 finding(s) at 324
[F] AATACA : 1 finding(s) at 1162
[F] AATAGA :
[F] AATATA :
[F] AATAAC : 1 finding(s) at 1006
[F] AATAAG :
[F] AATAAT :
[F] AATATT :
[F] AATAAA : 2 finding(s) at 7, 1597
[F] TTTGTA :

[F]:Forward

HNop 2

ORIGINAL

[F] GTAAGT : 1 finding(s) at 1615
[F] AATAAA : 1 finding(s) at 1678
[F] CATAAA :
[F] GATAAA : 1 finding(s) at 205
[F] TATAAA : 4 finding(s) at 148, 169, 190, 319
[F] ACTAAA :
[F] AGTAAA :
[F] ATTAAA :
[F] AAAAAA :
[F] AACAAA : 1 finding(s) at 1363
[F] AAGAAA : 1 finding(s) at 38
[F] AATCAA : 1 finding(s) at 295
[F] AATGAA : 1 finding(s) at 1546
[F] AATTAA : 1 finding(s) at 405
[F] AATACA : 2 finding(s) at 55, 1243
[F] AATAGA :
[F] AATATA : 1 finding(s) at 1071
[F] AATAAC : 2 finding(s) at 384, 1087
[F] AATAAG :
[F] AATAAT :
[F] AATATT : 1 finding(s) at 1578
[F] AATAAA : 1 finding(s) at 1678
[F] TTTGTA :

[F]:Forward

OPTIMIZED

[F] GTAAGT : 1 finding(s) at 784
[F] AATAAA : 1 finding(s) at 1678
[F] CATAAA :
[F] GATAAA : 1 finding(s) at 205
[F] TATAAA : 3 finding(s) at 148, 190, 319
[F] ACTAAA :
[F] AGTAAA :
[F] ATTAAA :
[F] AAAAAA :
[F] AACAAA : 1 finding(s) at 1363
[F] AAGAAA : 1 finding(s) at 38
[F] AATCAA : 1 finding(s) at 295
[F] AATGAA : 1 finding(s) at 1546
[F] AATTAA :
[F] AATACA : 1 finding(s) at 1243
[F] AATAGA :
[F] AATATA :
[F] AATAAC :
[F] AATAAG :
[F] AATAAT :
[F] AATATT :
[F] AATAAA : 1 finding(s) at 1678
[F] TTTGTA :

[F]:Forward

HNop 3

ORIGINAL

[F] GTAAGT : 1 finding(s) at 1546
[F] AATAAA : 1 finding(s) at 1609
[F] CATAAA :
[F] GATAAA : 1 finding(s) at 136
[F] TATAAA : 4 finding(s) at 79, 100, 121, 250
[F] ACTAAA :
[F] AGTAAA :
[F] ATTAAA :
[F] AAAAAA :
[F] AACAAA : 1 finding(s) at 1294

[F] AAGAAA :
[F] AATCAA : 1 finding(s) at 226
[F] AATGAA : 1 finding(s) at 1477
[F] AATTAA : 1 finding(s) at 336
[F] AATACA : 1 finding(s) at 1174
[F] AATAGA :
[F] AATATA : 1 finding(s) at 1002
[F] AATAAC : 2 finding(s) at 315, 1018
[F] AATAAG :
[F] AATAAT :
[F] AATATT : 1 finding(s) at 1509
[F] AATAAA : 1 finding(s) at 1609
[F] TTTGTA :

[F]:Forward

OPTIMIZED

[F] GTAAGT : 1 finding(s) at 992
[F] AATAAA :
[F] CATAAA :
[F] GATAAA :
[F] TATAAA : 4 finding(s) at 79, 250, 1072, 1122
[F] ACTAAA :
[F] AGTAAA :
[F] ATTAAA :
[F] AAAAAA :
[F] AACAAA :
[F] AAGAAA :
[F] AATCAA : 1 finding(s) at 336
[F] AATGAA : 1 finding(s) at 306
[F] AATTAA :
[F] AATACA : 4 finding(s) at 7, 1021, 1174, 1645
[F] AATAGA :
[F] AATATA :
[F] AATAAC :
[F] AATAAG :
[F] AATAAT : 1 finding(s) at 1018
[F] AATATT :
[F] AATAAA :
[F] TTTGTA :

[F]:Forward

HNop 4

ORIGINAL

[F] GTAAGT : 1 finding(s) at 1615
[F] AATAAA : 1 finding(s) at 1678
[F] CATAAA :
[F] GATAAA : 1 finding(s) at 205
[F] TATAAA : 4 finding(s) at 148, 169, 190, 319
[F] ACTAAA :
[F] AGTAAA :
[F] ATTAAA :
[F] AAAAAA :
[F] AACAAA : 1 finding(s) at 1363
[F] AAGAAA : 1 finding(s) at 38
[F] AATCAA : 1 finding(s) at 295
[F] AATGAA : 1 finding(s) at 1546
[F] AATTAA : 1 finding(s) at 405
[F] AATACA : 2 finding(s) at 55, 1243
[F] AATAGA :
[F] AATATA : 1 finding(s) at 1071
[F] AATAAC : 2 finding(s) at 384, 1087
[F] AATAAG :
[F] AATAAT :
[F] AATATT : 1 finding(s) at 1578
[F] AATAAA : 1 finding(s) at 1678
[F] TTTGTA :

[F]:Forward

OPTIMIZED

[F] GTAAGT : 1 finding(s) at 784
[F] AATAAA : 1 finding(s) at 1678
[F] CATAAA :
[F] GATAAA : 1 finding(s) at 205
[F] TATAAA : 3 finding(s) at 148, 190, 319
[F] ACTAAA :
[F] AGTAAA :
[F] ATTAAA :
[F] AAAAAA :
[F] AACAAA : 1 finding(s) at 1363
[F] AAGAAA : 1 finding(s) at 38
[F] AATCAA : 1 finding(s) at 295
[F] AATGAA : 1 finding(s) at 1546
[F] AATTAA :
[F] AATACA : 1 finding(s) at 1243
[F] AATAGA :
[F] AATATA :
[F] AATAAC :
[F] AATAAG :
[F] AATAAT :
[F] AATATT :
[F] AATAAA : 1 finding(s) at 1678
[F] TTTGTA :

[F]:Forward

POTENTIAL INTRON CRYTIC SPLICE SITES

HNop 1

ORIGINAL

[F] GCAGG :
[F] AGGTATGT :
[F] CAGG : 7 finding(s) at 177, 244, 488, 494, 632, 1351, 1653
[F] GTAAGT : 1 finding(s) at 1534
[F] GTACGT :
[F] AGGTA : 1 finding(s) at 393
[F] GTAAGT : 1 finding(s) at 1534
[F] AGGTGAG :
[F] GGGTGAG :
[F] AGGTAAGT :
[F] AGGTACGT :
[F] AGGTAGGT :
[F] AGGTATGT :
[F] AGGTCAGT :
[F] AGGTCCGT :
[F] AGGTCGGT :
[F] AGGTCTGT :
[F] AGGTGAGT :
[F] AGGTGCGT :
[F] AGGTGGGT :
[F] AGGTGTGT :
[F] AGGTTAGT :
[F] AGGTTCGT :
[F] AGGTTGGT :
[F] AGGTTTGT :

[F]:Forward

OPTIMIZED

[F] GCAGG : 1 finding(s) at 154
[F] AGGTATGT :

[F] CAGG : 8 finding(s) at 155, 189, 244, 632, 1351, 1471, 1653, 1692
[F] GTAAGT :
[F] GTACGT :
[F] AGGTA : 3 finding(s) at 156, 393, 1188
[F] GTAAGT :
[F] AGGTGAG :
[F] GGGTGAG :
[F] AGGTAAGT :
[F] AGGTACGT :
[F] AGGTAGGT :
[F] AGGTATGT :
[F] AGGTCAGT :
[F] AGGTCCGT :
[F] AGGTCGGT :
[F] AGGTCTGT :
[F] AGGTGAGT :
[F] AGGTGCGT :
[F] AGGTGGGT :
[F] AGGTGTGT :
[F] AGGTTAGT :
[F] AGGTTCGT :
[F] AGGTTGGT :
[F] AGGTTTGT :

[F]:Forward

HNop 2

ORIGINAL

[F] GCAGG :
[F] AGGTATGT :
[F] CAGG : 7 finding(s) at 258, 325, 569, 575, 713, 1432, 1734
[F] GTAAGT : 1 finding(s) at 1615
[F] GTACGT :
[F] AGGTA : 1 finding(s) at 474
[F] GTAAGT : 1 finding(s) at 1615
[F] AGGTGAG :
[F] GGGTGAG :
[F] AGGTAAGT :
[F] AGGTACGT :
[F] AGGTAGGT :
[F] AGGTATGT :
[F] AGGTCAGT :
[F] AGGTCCGT :
[F] AGGTCGGT :
[F] AGGTCTGT :
[F] AGGTGAGT :
[F] AGGTGCGT :
[F] AGGTGGGT :
[F] AGGTGTGT :
[F] AGGTTAGT :
[F] AGGTTCGT :
[F] AGGTTGGT :
[F] AGGTTTGT :

[F]:Forward

OPTIMIZED

[F] GCAGG : 1 finding(s) at 653
[F] AGGTATGT :
[F] CAGG : 6 finding(s) at 75, 258, 325, 654, 1108, 1415
[F] GTAAGT : 1 finding(s) at 784
[F] GTACGT : 1 finding(s) at 1289
[F] AGGTA : 2 finding(s) at 824, 1287
[F] GTAAGT : 1 finding(s) at 784
[F] AGGTGAG :
[F] GGGTGAG :
[F] AGGTAAGT :
[F] AGGTACGT : 1 finding(s) at 1287
[F] AGGTAGGT :
[F] AGGTATGT :
[F] AGGTCAGT :
[F] AGGTCCGT :
[F] AGGTCGGT :
[F] AGGTCTGT :
[F] AGGTGAGT :
[F] AGGTGCGT :
[F] AGGTGGGT :
[F] AGGTGTGT :
[F] AGGTTAGT :
[F] AGGTTCGT :
[F] AGGTTGGT :
[F] AGGTTTGT :

[F]:Forward

HNop 3

ORIGINAL

[F] GCAGG :
[F] AGGTATGT :
[F] CAGG : 8 finding(s) at 70, 189, 256, 500, 506, 644, 1363, 1665
[F] GTAAGT : 1 finding(s) at 1546
[F] GTACGT :
[F] AGGTA : 1 finding(s) at 405
[F] GTAAGT : 1 finding(s) at 1546
[F] AGGTGAG :
[F] GGGTGAG :
[F] AGGTAAGT :
[F] AGGTACGT :
[F] AGGTAGGT :
[F] AGGTATGT :
[F] AGGTCAGT :
[F] AGGTCCGT :
[F] AGGTCGGT :
[F] AGGTCTGT :
[F] AGGTGAGT :
[F] AGGTGCGT :
[F] AGGTGGGT :
[F] AGGTGTGT :
[F] AGGTTAGT :
[F] AGGTTCGT :
[F] AGGTTGGT :
[F] AGGTTTGT :

[F]:Forward

OPTIMIZED

[F] GCAGG :
[F] AGGTATGT :
[F] CAGG : 10 finding(s) at 189, 229, 466, 506, 896, 985, 1048, 1079, 1346, 1363
[F] GTAAGT : 1 finding(s) at 992
[F] GTACGT :
[F] AGGTA : 1 finding(s) at 1200
[F] GTAAGT : 1 finding(s) at 992
[F] AGGTGAG :
[F] GGGTGAG : 1 finding(s) at 1143
[F] AGGTAAGT :
[F] AGGTACGT :
[F] AGGTAGGT :
[F] AGGTATGT :
[F] AGGTCAGT :
[F] AGGTCCGT :
[F] AGGTCGGT :
[F] AGGTCTGT :
[F] AGGTGAGT :
[F] AGGTGCGT :
[F] AGGTGGGT :

[F] AGGTGTGT :
[F] AGGTTAGT :
[F] AGGTTCGT : 1 finding(s) at 1049
[F] AGGTTGGT :
[F] AGGTTTGT :

[F]:Forward

HNop 4

ORIGINAL

[F] GCAGG :
[F] AGGTATGT :
[F] CAGG : 7 finding(s) at 258, 325, 569, 575, 713, 1432, 1734
[F] GTAAGT : 1 finding(s) at 1615
[F] GTACGT :
[F] AGGTA : 1 finding(s) at 474
[F] GTAAGT : 1 finding(s) at 1615
[F] AGGTGAG :
[F] GGGTGAG :
[F] AGGTAAGT :
[F] AGGTACGT :
[F] AGGTAGGT :
[F] AGGTATGT :
[F] AGGTCAGT :
[F] AGGTCCGT :
[F] AGGTCGGT :
[F] AGGTCTGT :
[F] AGGTGAGT :
[F] AGGTGCGT :
[F] AGGTGGGT :
[F] AGGTGTGT :
[F] AGGTTAGT :
[F] AGGTTCGT :
[F] AGGTTGGT :
[F] AGGTTTGT :

[F]:Forward

OPTIMIZED

[F] GCAGG : 1 finding(s) at 653
[F] AGGTATGT :
[F] CAGG : 6 finding(s) at 75, 258, 325, 654, 1108, 1415
[F] GTAAGT : 1 finding(s) at 784
[F] GTACGT : 1 finding(s) at 1289
[F] AGGTA : 2 finding(s) at 824, 1287
[F] GTAAGT : 1 finding(s) at 784
[F] AGGTGAG :
[F] GGGTGAG :
[F] AGGTAAGT :
[F] AGGTACGT : 1 finding(s) at 1287
[F] AGGTAGGT :
[F] AGGTATGT :
[F] AGGTCAGT :
[F] AGGTCCGT :
[F] AGGTCGGT :
[F] AGGTCTGT :
[F] AGGTGAGT :
[F] AGGTGCGT :
[F] AGGTGGGT :
[F] AGGTGTGT :
[F] AGGTTAGT :
[F] AGGTTCGT :
[F] AGGTTGGT :
[F] AGGTTTGT :

[F]:Forward

RESTRICTION ENZYMES SITES

HNop 1

ORIGINAL

[F] (PacI) : 1 finding(s) at 1
[F] (HindIII) : 1 finding(s) at 1760
[F] (XhoI) : 1 finding(s) at 9
[F] (PstI) : 2 finding(s) at 15, 613
[F] (SpeI) : 1 finding(s) at 1766
[F] (AvrII) : 1 finding(s) at 1772

[F]:Forward

OPTIMIZED

[F] (PacI) : 1 finding(s) at 2
[F] (HindIII) : 1 finding(s) at 1761
[F] (XhoI) : 1 finding(s) at 10
[F] (PstI) : 1 finding(s) at 16
[F] (SpeI) : 1 finding(s) at 1767
[F] (AvrII) : 1 finding(s) at 1773

[F]:Forward

HNop 2

ORIGINAL

[F] (PacI) : 1 finding(s) at 1
[F] (HindIII) : 1 finding(s) at 1841
[F] (XhoI) : 1 finding(s) at 9
[F] (PstI) : 2 finding(s) at 15, 694
[F] (SpeI) : 1 finding(s) at 1847
[F] (AvrII) : 1 finding(s) at 1853

[F]:Forward

OPTIMIZED

[F] (PacI) : 1 finding(s) at 2
[F] (HindIII) : 1 finding(s) at 1842
[F] (XhoI) : 1 finding(s) at 10
[F] (PstI) : 1 finding(s) at 16
[F] (SpeI) : 1 finding(s) at 1848
[F] (AvrII) : 1 finding(s) at 1854

[F]:Forward

HNop 3

ORIGINAL
[F] (PacI) : 1 finding(s) at 1
[F] (HindIII) : 1 finding(s) at 1772
[F] (XhoI) : 1 finding(s) at 9
[F] (PstI) : 3 finding(s) at 15, 88, 625
[F] (SpeI) : 1 finding(s) at 1778
[F] (AvrII) : 1 finding(s) at 1784

[F]:Forward

OPTIMIZED
[F] (PacI) : 1 finding(s) at 2
[F] (HindIII) : 1 finding(s) at 1773
[F] (XhoI) : 1 finding(s) at 10
[F] (PstI) : 1 finding(s) at 16
[F] (SpeI) : 1 finding(s) at 1779
[F] (AvrII) : 1 finding(s) at 1785

[F]:Forward

HNop 4

ORIGINAL
[F] (PacI) : 1 finding(s) at 1
[F] (HindIII) : 1 finding(s) at 1818
[F] (XhoI) : 1 finding(s) at 9
[F] (PstI) : 2 finding(s) at 15, 671
[F] (SpeI) : 1 finding(s) at 1824
[F] (AvrII) : 1 finding(s) at 1830

[F]:Forward

OPTIMIZED
[F] (PacI) : 1 finding(s) at 2
[F] (HindIII) : 1 finding(s) at 1819
[F] (XhoI) : 1 finding(s) at 10
[F] (PstI) : 1 finding(s) at 16
[F] (SpeI) : 1 finding(s) at 1825
[F] (AvrII) : 1 finding(s) at 1831

[F]:Forward

ANNEX C

STATISTICAL ANALYSIS 1. Multifactorial ANOVA and t-test of all experiments with a significance of 95%. The values analized correspond to the raw values given by the reader and do not indicate the amount of expressed HN protein or TSP.

ANOVA for HN protein - Suma de Cuadrados Tipo III

Fuente	*Suma de Cuadrados*	*Gl*	*Cuadrado Medio*	*Razón-F*	*Valor-P*
EFECTOS PRINCIPALES					
A:DPI	19397,1	2	9698,54	54,83	0,0000
B:GENE	9202,8	3	3067,6	17,34	0,0000
C:SYSTEM	12419,1	2	6209,57	35,11	0,0000
INTERACCIONES					
AB	1650,49	6	275,081	1,56	0,1600
AC	3124,71	4	781,178	4,42	0,0018
BC	9262,66	6	1543,78	8,73	0,0000
RESIDUOS	53065,0	300	176,883		
TOTAL (CORREGIDO)	108122,	323			

Todas las razones-F se basan en el cuadrado medio del error residual

Tabla de Medias por Mínimos Cuadrados para HN protein con intervalos de confianza del 95,0%

			Error	*Límite*	*Límite*
Nivel	*Casos*	*Media*	*Est.*	*Inferior*	*Superior*
MEDIA GLOBAL	324	29,1675			
DPI					
FOUR	108	30,5063	1,27977	27,9878	33,0248
SIX	108	37,9033	1,27977	35,3848	40,4218
TWO	108	19,093	1,27977	16,5745	21,6115
GENE					
HNop1	81	36,552	1,47775	33,6439	39,46
HNop2	81	31,901	1,47775	28,993	34,8091
HNop3	81	24,5704	1,47775	21,6624	27,4785
HNop4	81	23,6467	1,47775	20,7386	26,5548
SYSTEM					
35S	108	21,0875	1,27977	18,569	23,6059
CMVva	108	30,2869	1,27977	27,7685	32,8054
TRBO	108	36,1282	1,27977	33,6098	38,6467
DPI por GENE					
FOUR,HNop1	27	36,947	2,55954	31,9101	41,984
FOUR,HNop2	27	32,9644	2,55954	27,9275	38,0013
FOUR,HNop3	27	25,5162	2,55954	20,4793	30,5532
FOUR,HNop4	27	26,5976	2,55954	21,5607	31,6345
SIX,HNop1	27	46,3419	2,55954	41,305	51,3789
SIX,HNop2	27	44,7356	2,55954	39,6987	49,7725
SIX,HNop3	27	30,3871	2,55954	25,3502	35,4241
SIX,HNop4	27	30,1486	2,55954	25,1117	35,1855
TWO,HNop1	27	26,367	2,55954	21,33	31,4039
TWO,HNop2	27	18,0031	2,55954	12,9662	23,04
TWO,HNop3	27	17,808	2,55954	12,771	22,8449
TWO,HNop4	27	14,1939	2,55954	9,15699	19,2309

DPI por SYSTEM					
FOUR,35S	36	20,4056	2,21662	16,0435	24,7677
FOUR,CMVva	36	34,3948	2,21662	30,0327	38,7569
FOUR,TRBO	36	36,7185	2,21662	32,3564	41,0806
SIX,35S	36	28,037	2,21662	23,6749	32,3991
SIX,CMVva	36	35,6872	2,21662	31,3251	40,0493
SIX,TRBO	36	49,9858	2,21662	45,6236	54,3479
TWO,35S	36	14,8197	2,21662	10,4576	19,1818
TWO,CMVva	36	20,7789	2,21662	16,4168	25,141
TWO,TRBO	36	21,6804	2,21662	17,3183	26,0425
GENE por SYSTEM					
HNop1,35S	27	25,526	2,55954	20,4891	30,563
HNop1,CMVva	27	34,7709	2,55954	29,7339	39,8078
HNop1,TRBO	27	49,359	2,55954	44,3221	54,396
HNop2,35S	27	19,1989	2,55954	14,162	24,2359
HNop2,CMVva	27	44,1186	2,55954	39,0816	49,1555
HNop2,TRBO	27	32,3856	2,55954	27,3487	37,4226
HNop3,35S	27	18,5667	2,55954	13,5298	23,6037
HNop3,CMVva	27	19,5235	2,55954	14,4865	24,5604
HNop3,TRBO	27	35,6211	2,55954	30,5842	40,658
HNop4,35S	27	21,0581	2,55954	16,0212	26,095
HNop4,CMVva	27	22,7349	2,55954	17,698	27,7718
HNop4,TRBO	27	27,1471	2,55954	22,1102	32,184

Pruebas de Múltiple Rangos para HN protein por DPI

Método: 95,0 porcentaje LSD

DPI	*Casos*	*Media LS*	*Sigma LS*	*Grupos Homogéneos*
TWO	108	19,093	1,27977	X
FOUR	108	30,5063	1,27977	X
SIX	108	37,9033	1,27977	X

Contraste	*Sig.*	*Diferencia*	*+/- Límites*
FOUR - SIX	*	-7,397	3,56165
FOUR - TWO	*	11,4133	3,56165
SIX - TWO	*	18,8103	3,56165

* indica una diferencia significativa.

Pruebas de Múltiple Rangos para HN protein por GENE

Método: 95,0 porcentaje LSD

GENE	*Casos*	*Media LS*	*Sigma LS*	*Grupos Homogéneos*
HNop4	81	23,6467	1,47775	X
HNop3	81	24,5704	1,47775	X
HNop2	81	31,901	1,47775	X
HNop1	81	36,552	1,47775	X

Contraste	*Sig.*	*Diferencia*	*+/- Límites*
HNop1 - HNop2	*	4,65094	4,11264
HNop1 - HNop3	*	11,9815	4,11264
HNop1 - HNop4	*	12,9053	4,11264
HNop2 - HNop3	*	7,33059	4,11264
HNop2 - HNop4	*	8,25433	4,11264
HNop3 - HNop4		0,923741	4,11264

* indica una diferencia significativa.

Pruebas de Múltiple Rangos para HN protein por SYSTEM

Método: 95,0 porcentaje LSD

SYSTEM	*Casos*	*Media LS*	*Sigma LS*	*Grupos Homogéneos*
35S	108	21,0875	1,27977	X
CMVva	108	30,2869	1,27977	X
TRBO	108	36,1282	1,27977	X

Contraste	*Sig.*	*Diferencia*	*+/- Límites*
35S - CMVva	*	-9,19949	3,56165
35S - TRBO	*	-15,0408	3,56165

CMVva - TRBO	*	-5,84128	3,56165

* indica una diferencia significativa.

ANOVA para TSP por GENE para hojas agroinfiltradas con CMVva

Fuente	*Suma de Cuadrados*	*Gl*	*Cuadrado Medio*	*Razón-F*	*Valor-P*
Entre grupos	0,020133	4	0,00503325	3,16	0,0161
Intra grupos	0,206811	130	0,00159085		
Total (Corr.)	0,226944	134			

Pruebas de Múltiple Rangos para TSP por GENE para hojas agroinfiltradas con CMVva

Método: 95,0 porcentaje LSD

GENE	*Casos*	*Media*	*Grupos Homogéneos*
Control	27	0,172037	X
HNop3	27	0,174444	X
HNop2	27	0,197111	X
HNop4	27	0,197148	X
HNop1	27	0,199852	X

Contraste	*Sig.*	*Diferencia*	*+/- Límites*
Control - HNop1	*	-0,0278148	0,0214763
Control - HNop2	*	-0,0250741	0,0214763
Control - HNop3		-0,00240741	0,0214763
Control - HNop4	*	-0,0251111	0,0214763
HNop1 - HNop2		0,00274074	0,0214763
HNop1 - HNop3	*	0,0254074	0,0214763
HNop1 - HNop4		0,0027037	0,0214763
HNop2 - HNop3	*	0,0226667	0,0214763
HNop2 - HNop4		-0,000037037	0,0214763
HNop3 - HNop4	*	-0,0227037	0,0214763

* indica una diferencia significativa.

ANOVA para TSP por DPI para hojas agroinfiltradas con CMVva

Fuente	*Suma de Cuadrados*	*Gl*	*Cuadrado Medio*	*Razón-F*	*Valor-P*
Entre grupos	0,0598224	2	0,0299112	23,63	0,0000
Intra grupos	0,167122	132	0,00126607		
Total (Corr.)	0,226944	134			

Pruebas de Múltiple Rangos para TSP por DPI para hojas agroinfiltradas con CMVva

Método: 95,0 porcentaje LSD

DPI	*Casos*	*Media*	*Grupos Homogéneos*
SIX	45	0,159933	X
TWO	45	0,193911	X
FOUR	45	0,210511	X

Contraste	*Sig.*	*Diferencia*	*+/- Límites*
FOUR - SIX	*	0,0505778	0,0148384
FOUR - TWO	*	0,0166	0,0148384
SIX - TWO	*	-0,0339778	0,0148384

* indica una diferencia significativa.

ANOVA para TSP por GENE para hojas agroinfiltradas con TRBO

Fuente	*Suma de Cuadrados*	*Gl*	*Cuadrado Medio*	*Razón-F*	*Valor-P*
Entre grupos	0,239206	4	0,0598016	7,43	0,0000
Intra grupos	1,04574	130	0,00804415		
Total (Corr.)	1,28495	134			

Pruebas de Múltiple Rangos para TSP por GENE para hojas agroinfiltradas con TRBO

Método: 95,0 porcentaje LSD

B.GENE	*Casos*	*Media*	*Grupos Homogéneos*
Control	27	0,15837	X
HNop1	27	0,236852	X
HNop3	27	0,258852	X
HNop2	27	0,261481	X
HNop4	27	0,277148	X

Contraste	*Sig.*	*Diferencia*	*+/- Límites*
Control - HNop1	*	-0,0784815	0,048293

Control - HNop2	*	-0,103111	0,048293
Control - HNop3	*	-0,100481	0,048293
Control - HNop4	*	-0,118778	0,048293
HNop1 - HNop2		-0,0246296	0,048293
HNop1 - HNop3		-0,022	0,048293
HNop1 - HNop4		-0,0402963	0,048293
HNop2 - HNop3		0,00262963	0,048293
HNop2 - HNop4		-0,0156667	0,048293
HNop3 - HNop4		-0,0182963	0,048293

* indica una diferencia significativa.

ANOVA para TSP por DPI para hojas agroinfiltradas con TRBO

Fuente	*Suma de Cuadrados*	*Gl*	*Cuadrado Medio*	*Razón-F*	*Valor-P*
Entre grupos	0,51182	2	0,25591	43,69	0,0000
Intra grupos	0,773125	132	0,00585701		
Total (Corr.)	1,28495	134			

Pruebas de Múltiple Rangos para TSP por DPI para hojas agroinfiltradas con TRBO

Método: 95,0 porcentaje LSD

B.DPI	*Casos*	*Media*	*Grupos Homogéneos*
SIX	45	0,163267	X
FOUR	45	0,238267	X
TWO	45	0,314089	X

Contraste	*Sig.*	*Diferencia*	*+/- Límites*
FOUR - SIX	*	0,075	0,0319151
FOUR - TWO	*	-0,0758222	0,0319151
SIX - TWO	*	-0,150822	0,0319151

* indica una diferencia significativa.

ANOVA para TSP por GENE para hojas agroinfiltradas con 35S

Fuente	*Suma de Cuadrados*	*Gl*	*Cuadrado Medio*	*Razón-F*	*Valor-P*
Entre grupos	0,0326637	4	0,00816591	3,36	0,0119
Intra grupos	0,31627	130	0,00243285		
Total (Corr.)	0,348934	134			

Pruebas de Múltiple Rangos para TSP por GENE para hojas agroinfiltradas con 35S

Método: 95,0 porcentaje LSD

C.GENE	*Casos*	*Media*	*Grupos Homogéneos*
Control	27	0,155444	X
HNop1	27	0,177481	XX
HNop3	27	0,181963	XX
HNop2	27	0,186741	X
HNop4	27	0,203593	X

Contraste	*Sig.*	*Diferencia*	*+/- Límites*
Control - HNop1		-0,022037	0,0265584
Control - HNop2	*	-0,0312963	0,0265584
Control - HNop3		-0,0265185	0,0265584
Control - HNop4	*	-0,0481481	0,0265584
HNop1 - HNop2		-0,00925926	0,0265584
HNop1 - HNop3		-0,00448148	0,0265584
HNop1 - HNop4		-0,0261111	0,0265584
HNop2 - HNop3		0,00477778	0,0265584
HNop2 - HNop4		-0,0168519	0,0265584
HNop3 - HNop4		-0,0216296	0,0265584

* indica una diferencia significativa.

ANOVA para TSP por DPI para hojas agroinfiltradas con 35S

Fuente	*Suma de Cuadrados*	*Gl*	*Cuadrado Medio*	*Razón-F*	*Valor-P*
Entre grupos	0,241068	2	0,120534	147,50	0,0000
Intra grupos	0,107865	132	0,000817162		
Total (Corr.)	0,348934	134			

Pruebas de Múltiple Rangos para TSP por DPI para hojas agroinfiltradas con 35S

Método: 95,0 porcentaje LSD

C.DPI	*Casos*	*Media*	*Grupos Homogéneos*
FOUR	45	0,143311	X
SIX	45	0,159778	X
TWO	45	0,240044	X

Contraste	*Sig.*	*Diferencia*	*+/- Límites*
FOUR - SIX	*	-0,0164667	0,011921
FOUR - TWO	*	-0,0967333	0,011921
SIX - TWO	*	-0,0802667	0,011921

* indica una diferencia significativa.