ABSTRACT

Influenza Hemagglutinin Expression in *Nicotiana tabacum* and *Nicotiana benthamiana*

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Influenza infects millions of people each year. Hemagglutinin (HA) is the major surface protein of the influenza virus and the best target as a vaccine candidate. Transgenic plants have significant potential for proper expression of HA to serve as an influenza vaccine.

An HA3 DNA construct for insertion into plants was developed using the binary vector, pE1802. *Agrobacterium tumefaciens* was transformed with the plasmid and both *Nicotiana tabacum* or *Nicotiana benthamiana* were transformed. To screen for HA3 expression, mRNA was isolated from first generation plants. Second generation plants were successfully screened for protein production using immunoblots to verify HA3 protein expression.

A tobacco mosaic virus (TMV) vector (pJL36) was also produced expressing HA3. Recombinant constructs of the TMV vector were successfully screened using PCR. This work indicates the potential use of HA3-expressing TMV vector for transformation into *A. tumefaciens,* infection into *N. benthamiana* and the expression of gene products in these systems.

Influenza Hemagglutinin Expression in *Nicotiana tabacum* and *Nicotiana benthamiana*

by

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A Thesis

Approved by the Department of Biology

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Robert D. Doyle, Ph.D., Chairperson

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CHAPTER ONE

Introduction

Influenza Virus

Influenza virus, from the family Orthomyxoviridae, includes three clades, A, B, and C (Hampson and Mackenzie 2006, Wong and Yuen 2006). Only influenza A causes severe disease, epidemics, and pandemics. Influenza C only causes a common cold-like illness (Black and Armstrong 2006, Hampson 2002) while influenza B, because it diverged and evolved uniquely as a human pathogen a considerable time ago, only causes minor epidemics (Black and Armstrong 2006, Hampson and Mackenzie 2006, Webster et al. 1992). Influenza A virus is one of the main infectious disease threats to humans (Cox and Subbarao 2000) and its infections have caused the World Health Organization to set up a program to monitor influenza virus outbreaks around the world (Potter 2001, Stohr 2003).

Influenza viruses are usually spread by inhalation of infectious droplets, aerosolized by either sneezing or coughing, or by coming into contact with surfaces that are contaminated with these infectious droplets (Bell 2006, Bean at al. 1982, Bridges et al. 2003, Chin 2000). Once inhaled, the virus rapidly infects cells, causing symptoms of fever, myalgia, headaches, sore throat, and cough (Mandell et al. 2005). These symptoms usually disappear in about a week, but pneumonia, respiratory failure, heart failure, and death occasionally occur (Black and Armstrong 2006).

Influenza A virus causes annual epidemics and every ten to fifty years, at unpredictable intervals, causes major pandemics (Dowdle 2006, Hampson and

Mackenzie 2006, Potter 2001, Taubenberger and Morens 2006). During annual epidemics 10% of the population may be infected with the virus (Christie 1987), with significantly more individuals infected during pandemics. According to the World Health Organization (WHO 2005), in order for a pandemic to occur three conditions must be met: a new virus subtype to which humans have little or no immunity must emerge, the new virus must be able to replicate in humans and cause serious illness, and the new virus must be easily transmitted from person to person.

In the past 300 years ten pandemics of influenza virus have occurred (Potter 1998, Potter 2001). The three main pandemics of the last century were the Asian influenza pandemic of 1957, the Hong Kong influenza pandemic of 1968 to 1969, and the Spanish influenza pandemic of 1918 to 1919 (Hampson and Mackenzie 2006, Wong and Yuen 2006). The Asian and Hong Kong influenza pandemics each killed approximately two million people (Hampson and Mackenzie 2006). Notably, the Spanish influenza pandemic has been called one of the top three plagues in history (Starr 1976). It killed an estimated fifty million people and infected 50% of the world's population (Crosby 1976, Hampson and Mackenzie 2006). Until recently the Spanish pandemic was believed to be caused by what researchers term antigenic shift, occurring when two different strains of influenza combine to form a new subtype containing surface antigens from both original strains (Black and Armstrong 2006, Wong and Yuen 2006). This term is specific to influenza; in other viral systems it is known as viral shift or reassortment (Webster and Laver 1971). Recent evidence shows, however, that this virus was derived from an avian influenza virus (Taubenberger at al. 2005, Tumpey at al. 2005), prompting scientists to predict that the next major pandemic could be caused by H5N1, an avian disease, that,

thus far, upon infecting humans has been lethal 60% of the time (Black and Armsrong 2006, Wong and Yuen 2006).

Influenza's potent antigenic variation from the processes of drift (Verhoeyen et al. 1980) and shift (Webster et al. 1982) enables these reoccurring epidemics. Antigenic drift has been defined as the accumulation of mutations in the genetic makeup of the influenza virus (Earn et al. 2002, Hampson 2002). Because RNA polymerase has no proofreading mechanism, influenza readily acquires mutations, since the virus cannot proofread its RNA for errors (Black and Armstrong 2006, Webster et al. 1992). Since these accumulated mutations selectively permit influenza to partially evade a host's immune system (Hampson 2002, Potter 2001, Wong and Yuen 2006), influenza antigenic drift has been studied extensively (Bush et al. 1999, Fitch et al. 1997, Mandell et al. 2005, Smith et al. 2004). A single amino acid change is sometimes sufficient to alter receptor binding specificity and allow for antigenic drift (Gambaryan et al. 2006, Matrosovich et al. 1997). There is evidence that antigenic shift, more so than antigenic drift, enables influenza to evade the human immune system.

Influenza's facile adaptability presents a significant obstacle to the design of a universal influenza vaccine (Fitch et al. 1997, Francis et al. 1947). Current vaccine approaches against influenza use inactivated or subunit influenza virus grown in embryo chicken cells (Kodihalli et al. 1995). Among the limitations of these approaches are the expense and logistic difficulty of producing vaccine for the entire world population (Emanuel and Wertheimer 2006). Other strategies to achieve protective immunity include peptide vaccination (Simeckova-Rosenberg et al. 1995), DNA vaccination (Ulmer et al. 1993), and recombinant vaccinia virus (Bender et al. 1996). Currently the

best alternative approach appears to be the use of recombinant influenza surface proteins (Musiychuk et al. 2007, Powers et al. 1995).

As a member of the Orthomyxoviridae family, influenza is a single stranded minus-sense RNA virus with a segmented genome (Wong and Yuen 2006). The eight RNA segments of the Influenza A virus genome encode eleven viral proteins, including the polymerase proteins (PB1, PB2, PA, and PB1-F2), nucleocapsid protein, hemagglutinin, neuraminidase, matrix proteins (M1,M2) and nonstructural proteins (NS1,NS2) (Hampson and Mackenzie 2006, Potter 2001, Wong and Yuen 2006). PB1-F2 causes cellular apoptosis by acting on the host cell's mitochondria (Chanturiya et al. 2004).

The surface of influenza consists of three proteins: M2, neuraminidase (NA), and hemagglutinin (HA) (Flint et al. 2004). M2 forms an ion channel used for pH-dependent dissociation of matrix proteins from the nucleocapsid during viral uncoating and pH changes occurring across the trans-Golgi network during production of HA proteins (Wong and Yuen 2006). M2, due to its relatively small concentration, does not elicit a strong enough immune system response when used in a vaccine to neutralize the infection (Slepushkin et al. 1995). NA facilitates the spread of influenza by cleaving glycosidic linkages to sialic host cells (Hampson and Mackenzie 2006, Wong and Yuen 2006). Whereas NA as a vaccine, is unable to stop infection, it does limit viral spread (Saelens et al. 1999, Sampson 2002). HA is responsible for virus attachment to the cell by binding to sialic acid receptors (Hampson and Mackenzie 2006, Wong and Yuen 2006). In order for the virus to be infectious, HA must be cleaved after translation by a trypsin-like protein into HA1 and HA2 (Webster at al. 1992). Because they are the major

antigenic determinants of influenza, HA and NA serve as the basis for subtype classification (Hampson and Mackenzie 2006, Wong and Yuen 2006). There are sixteen distinct HA subtypes (named H1-H16) and nine distinct NA subtypes (named N1-N9) (Fouchier et al. 2005, Hampson and Mackenzie 2006, Wong and Yuen 2006).

HA, as the major viral surface protein, is the influenza protein recognized by a majority of the body's antibodies in response to infection (Potter and Oxford 1979, Webster et al. 1982, Wong and Yuen 2006). Anti-HA antibodies prevent the low-pH-induced conformational change of HA requisite for influenza virion binding to a host cell (Bush et al. 1999). Because of this potential for viral neutralization, HA is the predominant focus of efforts to make an effective influenza vaccine.

Vaccine Production in Transgenic Plants

Current methods for vaccine production use live attenuated or inactivated viruses, but difficulties and risks involved in producing large amounts of vaccines by these approaches have triggered an interest in developing viral protein subunit vaccines (Varsani et al. 2003). Protein expression of subunit vaccines in bacteria, yeast, and mammalian cell systems has become popular, but these systems each present problematic limitations. Mammalian cell culture systems feature high costs, particular reagent requirements, and considerable technical skill. Bacterial expression is relatively inexpensive, but both post-translational modification variation and quality control issues decrease its appeal. Proteins from yeast expression systems generally also possess non-wild type post-translational modifications. Moreover, all of these methods require expensive technical equipment and facilities (Baneyx 1999, Butler et al. 2003, Hannig

and Makrides 1998, Harashima 1994, Hunt 2005, Makrides 1996, Palomares et al. 2004, Roodveldt et al. 1995, Streatfield and Howard 2003, Varsani et al. 2003).

Limitations in and expense of current vaccine production methods have driven exploration of alternatives, including transgenic plants as a potentially viable option. Transgenic plants offer several advantages over traditional methods to produce vaccines: these include their ability to lower the cost of raw materials, high production capability, ability to produce multiple vaccines in one plant, convenient storage of raw materials, reduced need to keep the vaccine refrigerated or frozen during transport, convenience of delivery in food or feed type products, reduced concerns over human pathogen contamination in vaccine preparations, elimination of cost for syringes and needles, reduced need of medical assistance to administer the vaccine, and elimination of concern over blood-borne diseases through needle reuse (Arntzen et al. 1994, Biemelt et al. 2003, Kusnadi et al. 1997, Kusnadi at al. 1998, Lamphear et al. 2002, Mason and Arntzen 1995, Musiychuk et al. 2007, Satyavathi et al. 2003, Streatfield and Howard 2003, Varsani et al. 2003, Webster at al. 2005, Whitelam 1995). Transgenic plants also have the advantage of offering a potential permanent source of the vaccine via seed production. Transgenic plants can also produce protein with post-translational modifications similar to those of higher eukaryotes that other traditional systems cannot (Streatfield and Howard 2003). Glycosylation patterns of mammalian proteins expressed in transgenic plants are correct, although different carbohydrate side chains are used (Bardor et al. 1999). Complex glycans of plants are usually smaller than those of animals, since glycans expressed in plants lack sialic acid (Faye et al. 1993). If the transgenic plant is a food-based crop that

can confer immunity orally, an additional advantage is that timely and costly purification steps can be eliminated (Hood and Jilka 1999, Streatfield and Howard 2003).

A major potential limitation of using plants to express foreign proteins, however, is a typical low protein expression that sometimes is insufficient to provide protection (Daniell et al. 2001, Daniell et al. 2002) Transgene expression levels are unpredictable and vary among transformants (Finnegan and McElroy 1994). This limitation requires optimization of the transgenic plant expression system in order to express sufficient protein to confer protection (Biemelt et al. 2003). A variety of strategies have been proposed to increase expression of foreign proteins in transgenic plants, including codon optimization, insertion of 5'-or 3'-untranslated regions to increase mRNA stability, modification of plant promoters for transcription of introduced genes, introduction of a 5'-untranslated tobacco mosaic virus sequence or other enhancer of translation, and the use of a Cauliflower mosaic virus 35S-dual enhancer promoter (Christensen et al. 2001, Mason et al. 1992, Mor et al. 1998, Richter et al. 2000).

In the last decade several proteins with potential for use as pharmaceuticals or vaccines have been expressed in plants (Carter and Langridge 2002, Daniell et al. 2001, Giddings et al. 2000, Jilka et al. 1999, Korban 2002, Kusnadi et al. 1997, Stoger et al. 2002, Twyman et al. 2005). For example, the B subunit of the heat labile toxin of enterotoxigenic strains of *Escherichia coli* has been expressed in both potato tubers (Lauterslager at al. 2001, Mason et al. 1998) and corn seed (Chikwamba et al. 2002, Streatfield et al. 2003). The hepatitis B surface antigen has been expressed in transgenic potatoes (Richter et al. 2000). The VP1 protein of foot and mouth disease has been expressed in transgenic alfalfa plants (Wigdorovitz et al. 1999). The spike protein of

swine-transmissible gastroenteritis coronavirus has been expressed in transgenic potato plants (Gomez et al. 2000). A transgenic pigeon pea expressing the Hemagglutinin protein of Rinderpest virus has been developed (Satyavathi et al. 2003), along with a transgenic peanut expressing the same protein (Khandelwal et al. 2003). Both the L1 protein of human papillomavirus type 16 and the Norwalk virus capsid protein have been successfully expressed in transgenic tobacco plants and potato plants (Biemelt et al. 2003, Liu et al. 2005, Mason et al. 1996). The surface-exposed Hemagglutinin protein from measles virus has been expressed in transgenic tobacco plants (Huang et al. 2001) and in transgenic carrots (Marquet-Blouin et al. 2003). Several plant-produced antigens induce cellular and humoral responses, conferring protection against challenge in mouse model systems (Arakawa et al. 1998, Brennan et al. 1999, Carrillo et al. 1998, Haq et al. 1995, Kapusta et al. 1999, Mason et al. 1996, Modelska et al. 1998, Satyavathi et al. 2003, Streatfield and Howard 2003, Thanavala et al. 1995, Wigdorovitz et al. 1999a, Wigdorowitz et al. 1999b), and several plant-produced vaccine candidates have completed phase I clinical trials (Tacket et al. 1998, Tacket et al. 2000, Tacket et al. 2004, Thanavala et al. 2005, Yusibov et al. 2002). Several different plant-produced antigens, administered orally as vaccines, have induced a protective immune response in humans and mice (Daniell et al. 2001, Huang et al. 2001, Tacket et al. 1998). Notably, 100-fold more antigen is required via the oral route than would be needed with vaccines delivered by injection (Streatfield and Howard 2003).

Several different strategies are available to express a desired protein in transgenic plants. These strategies include nuclear transformation (Hood and Jilka 1999), chloroplast transformation (Kumar and Daniell 2004), and infection with RNA viral

vectors (Yusibov et al. 2006). Nuclear transformants generally have low levels of expression and long lead times (Musiychuk et al. 2007). The technique of chloroplast transformation yields high protein expression levels but is limited to a few plant species, has limited post-translational modification, and also has a long lead time (Kumar and Daniell 2004). In contrast, viral vectors have short lead times, capacitiy for rapid bulk-up, facile molecular manipulation to fuse peptides to the viral coat protein for greater protein yield and immunogenicity, and high protein expression levels (Yusibov et al. 2002, Yusibov et al. 2006). An example of fusing peptides to inserted proteins to allow for greater protein expression is the mountain cedar antigen Jun a 3. Attachment of the native signal peptide Jun a 3 to a target protein causes the desired protein to accumulate in the interstitial space of the plant (Krebitz et al. 2003, M. Moehnke, personal communication).

Tobacco Mosaic Virus (TMV)

The identification of viruses tropic for various taxonomic families of plants and the molecular manipulation of several of these viruses to express proteins of inserted genes makes plant viral systems a facile approach to generate transgenic plants (Kearney et al. 1995). Plants with a virus-based vector inserted into their genomes have been shown to produce a range of valuable functional peptides and proteins (Santa Cruz et al. 1995). A virus-based vector consists of an engineered virus capable of expressing recombinant genes whereby the virus rapidly amplifies the inserted genes. When a virus-based vector with an inserted gene is transformed into a plant, it results in expression of the target protein throughout the entire plant (Scholthof et al. 1996). Plant viruses are well suited as transient expression vectors because of characteristics such as high-level

expression of introduced genes, rapid accumulation of expressed exogenous proteins, and ready purification of these proteins from the host plant (Yusibov et al. 1999). These traits make viral-based plant expression a potentially cost-effective source of therapeutic reagents (Yusibov et al. 1999). One to two weeks after infection, recombinant proteins can be isolated from the leaves of the plant (Kumagai et al. 2000). However, this method requires that viral transformation be performed iteratively—each generation, as compared to only once with transgenic plants.

One important source of plant viral vectors has been tobacco mosaic virus (TMV). TMV was initially identified and named 'tobacco mosaic' by Adolf Mayer (Mayer 1886), with important additional work being performed by Dimitrii Ivanowski (Ivanowski 1892). However, the first person to report that TMV could infect only growing and dividing parts of the plant, describe the flow of TMV inside the plant, discover the two modes of infection, local and general, classify symptoms of the tobacco mosaic disease, and characterize the development of malformed leaf tissues due to the virus was Martinus Willem Beijerinck (Beijerinck 1898). The TMV crystal structure was elucidated in 1935 (Stanley 1935). TMV was the first virus to have its rod-like morphology visualized by electron microscopy (Kausche et al. 1939). TMV RNA was sequenced in 1982, and it was the first plant viral genome to be sequenced (Goelet et al. 1982). TMV has a broad host range, able to infect a wide variety of plants (Harrison and Wilson 1999).

TMV, a tobamovirus, is a single-stranded, positive sense RNA virus (Goelet et al. 1982). Its genome consists of genomic RNA and sub-genomic RNA (Fig. 1). The viral genomic RNA codes for two replicase-associated proteins (Ishikawa et al. 1986) and a movement protein (30 kDa) (MP) (Deom et al. 1987). The sub-genomic RNA codes for a

coat protein (17.5 kDa) (CP) (Dawson et al. 1988). The genome of TMV employs a read- through of the stop codon to generate the viral polymerase (Dawson and Lehto 1990). The normal replicase protein at 126 kDa and the read-through replicase

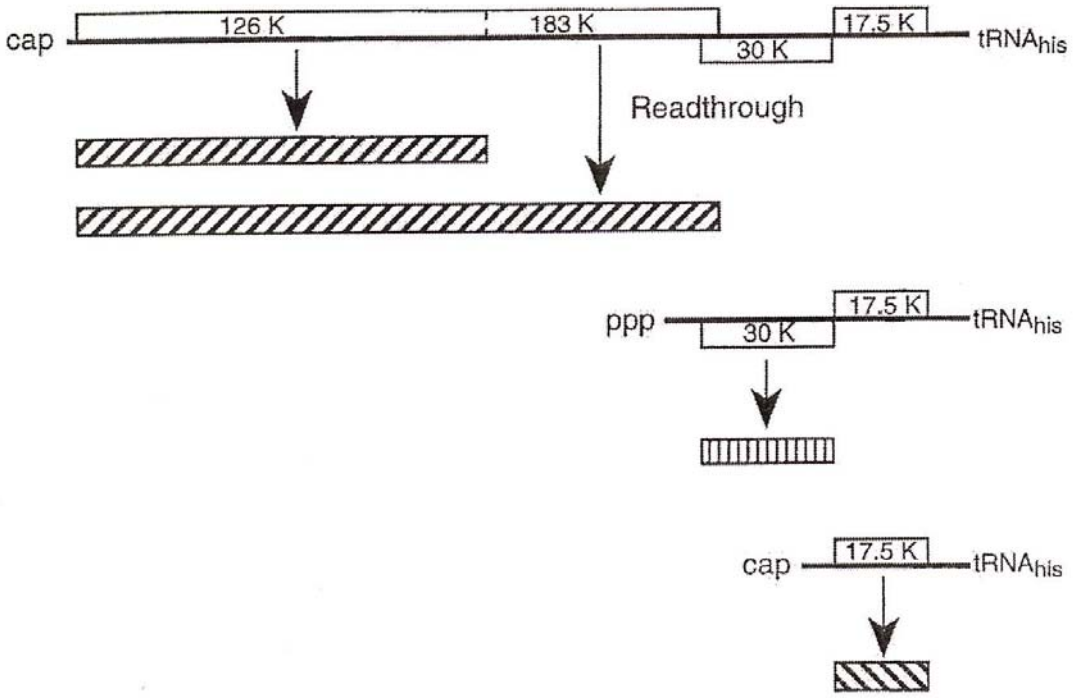

Figure 1. Replication of Genomic and Sub-genomic RNA of the Tobacco Mosaic Virus (TMV) (Shaw 1996). The viral genomic RNA codes for two replicase-associated proteins (Ishikawa et al. 1986) and a movement protein (30 kDa) (MP) (Deom et al. 1987). The sub-genomic RNA codes for a coat protein (17.5 kDa) (CP) (Dawson et al. 1988). The genome of TMV employs a read-through of the stop codon to generate the viral polymerase (Dawson and Lehto 1990). The normal replicase protein at 126 kDa and the read-through replicase protein at 183 kDa comprise the RNA dependent RNA polymerase which synthesizes a minus-sense copy of the TMV genome (Dawson and Lehto 1990). The resulting copies are used to replicate plus-sense genomes and the sub-genomic MP and CP mRNAs. The MP modifies plasmodesmatal junctions, is required for cell-to-cell movement of viral RNA genomes, and is produced early in the infection cycle of TMV (Deom et al. 1990, Deom et al. 1992). The CP is a structural protein required for encapsidation of the viral genome and systemic movement of the virus through the vascular system of the plant (Dawson et al. 1988). The CP spontaneously initiates encapsidation of the viral genome at an RNA sequence within the MP encoding region, called the origin of assembly (Dawson and Lehto1990).

protein at 183 kDa comprise the RNA dependent RNA polymerase which synthesizes a minus-sense copy of the TMV genome (Dawson and Lehto 1990). The resulting copies are used to replicate plus-sense genomes and the sub-genomic MP and CP mRNAs.

The MP modifies plasmodesmatal junctions, is required for cell-to-cell movement of viral RNA genomes, and is produced early in the infection cycle of TMV (Deom et al. 1990, Deom et al. 1992). As the infection cycle continues, production shifts from MP mRNA to genomic RNA and CP mRNA. CP is a structural protein required for encapsidation of the viral genome and systemic movement of the virus through the vascular system of the plant (Dawson et al. 1988). The CP spontaneously initiates encapsidation of the viral genome at an RNA sequence within the MP encoding region, called the origin of assembly (Dawson and Lehto1990). The genome RNA of TMV has a 3' untranslated region (UTR) that has a tRNA-like structure and pseudoknots (Zeenko et al. 2002). Pseudoknots, phylogenetically conserved genetic sequences, assist in correct translation of the TMV viral genome (Leathers et al. 1993).

Two approaches have been used to develop TMV as an expression vector. The first method uses viral RNA transcripts from cDNA clones to serve as infectious templates for inoculation of plants (Dawson et al. 1986, Pogue 1998). The second method, introduces viral cDNA by plant agro-inoculation. The cDNA of TMV is first sub-cloned into an agro-bacterial-based plasmid of *Agrobacterium tumefaciens*. Plants are then transfected by agro-inoculation with the recombinant *A. tumefaciens* (Turpen et al. 1993).

Because of the ease of transformation and regeneration of this species, antigens and proteins are often expressed in tobacco plants (Satyavathi et al. 2003). Additionally,

there is no natural vector to transmit TMV into crop plants, and it is less biohazardous than other virus vectors (Pogue 1998). TMV replicates extra-chromosomally and expresses high levels of the target protein throughout the plant (Casper and Holt 1996). Infections with TMV continue throughout the lifetime of the plant. However, due to the high toxic levels of alkaloids in tobacco leaves, substantial purification of tobacco-derived antigens is necessary in order to conduct studies involving animal feedings (Satyavathi et al. 2003).

CHAPTER TWO

Materials and Methods

Construction of a Functional Bacterial Plant Expression Vector with the Insert HA.

To construct a Hemagglutinin3 (HA3) expression plasmid, influenza HA3 cDNA (a gift of X. Saelens, University of Ghent) was sub-cloned into plant expression vector pE1802 (Ni et al. 1995) (Fig 2), and the resultant construct was inserted into the genome

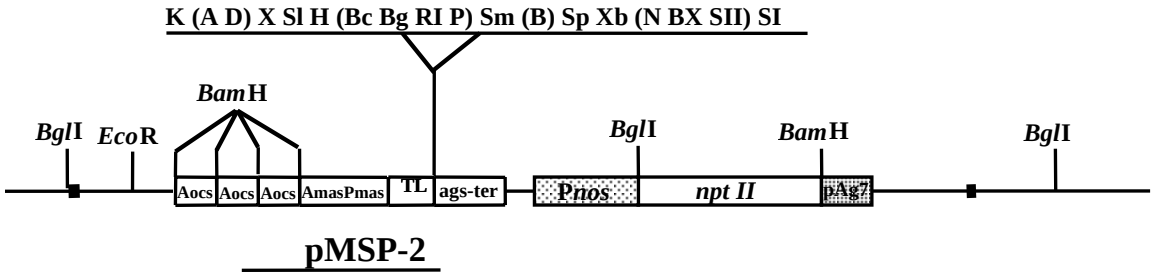

Figure 2. Relevant Regions of the Cloning Vector pE1802. Vector pE1802 is a binary plant transformation vector, with the T-DNA region shown here, comprising the kanamycin resistance cassette and the super promoter cassette (Ni et al. 1995). The super promoter cassette consists of the three Aocs genes and the AmasPmas genes. Aocs stands for octopine synthase activator, Amas stands for mannopine synthase activator, and Pmas stands for mannopine synthase promoter (Ni et al. 1995). TL is the translational leader, which is derived from a picorna-like virus (a potyvirus) Tobacco etch virus (Ni et al. 1995). The multiple cloning site (MCS) contains a variety of restriction enzyme sites including *KpnI* and *SacI*, the sites that will be used for this project. Ags-ter is the terminator from Agrobacterium tumefaciens. pMSP-2 stands for putative microspore specific promoter 2 which codes for the gene At5g46795 and upregulates gene expression (Honys et al. 2006). Pnos stands for nopaline synthase promoter, npt II stands for Neomycin Phosphotransferase II which confers kanamycin resistance, and pAg7 is another *Agrobacterium tumefaciens* promoter (Ni et al. 1995).

of *Nicotiana benthamina* or *Nicotiana tabacum*. Before subcloning sequence encoding two individual signal peptides, either a native HA signal peptide or a Jun a 3 signal peptide, was attached 5' to the open reading frame (ORF) of HA3 by PCR primer

addition. Jun a 3 is a juniper allergen that expresses strongly to the *N. benthamina* interstitial fluid (Moenke & Kearney, unpublished data).

Additionally, restriction enzyme sites *KpnI* at the 5' end and site at the 3' end of the HA3 ORF were added by PCR addition for purposes of cloning into pE1802 (Fig. 3). The cloned expression construct comprises 1.8 kb of HA signal peptide sequence, the HA3 coding sequence and the *KpnI* and *SacI* restriction enzymes sites. Colonies resulting from ligation of the PCR product followed by transformation and growth in *E. coli* were screened by plasmid isolation followed by PCR analysis of a 300 base pair product from the 3' end of the HA3 gene. A positive clone, HA3/pE1802, was selected and transformed into *Agrobacterium tumefaciens.* The plasmid-containing bacteria was introduced into *N. tabacum* as well as *N. benthamiana* by *A. tumefaciens* inoculation. Use of *Agrobacterium* binary plasmids, based on the tumor-inducing plasmid *A. tumefaciens*, is a standard tool for generation of transgenic plants (Gelvin 2000).

Generation of Transgenic Plant Lines Expressing HA3 Protein

Electrocompetent *A. tumefaciens* bacterial cells were transformed with plasmid (Biemelt et al. 2003, Huang et al. 2001, Liu et al. 2005, Marquet-Blouin et al. 2003) containing either HA leader plus HA3 sequence or Jun a 3 leader plus HA3 sequence and cultured in L Broth. Leaves of *N. tabacum* and *N. benthamiana* were cut into one cm square pieces and dipped into the *A. tumefaciens* culture. After 15 minutes incubation at room temperature with rocking, they were transferred to agar plates and cultured for two days at room temperature, followed by transfer to antibiotic kanamycin agar media.

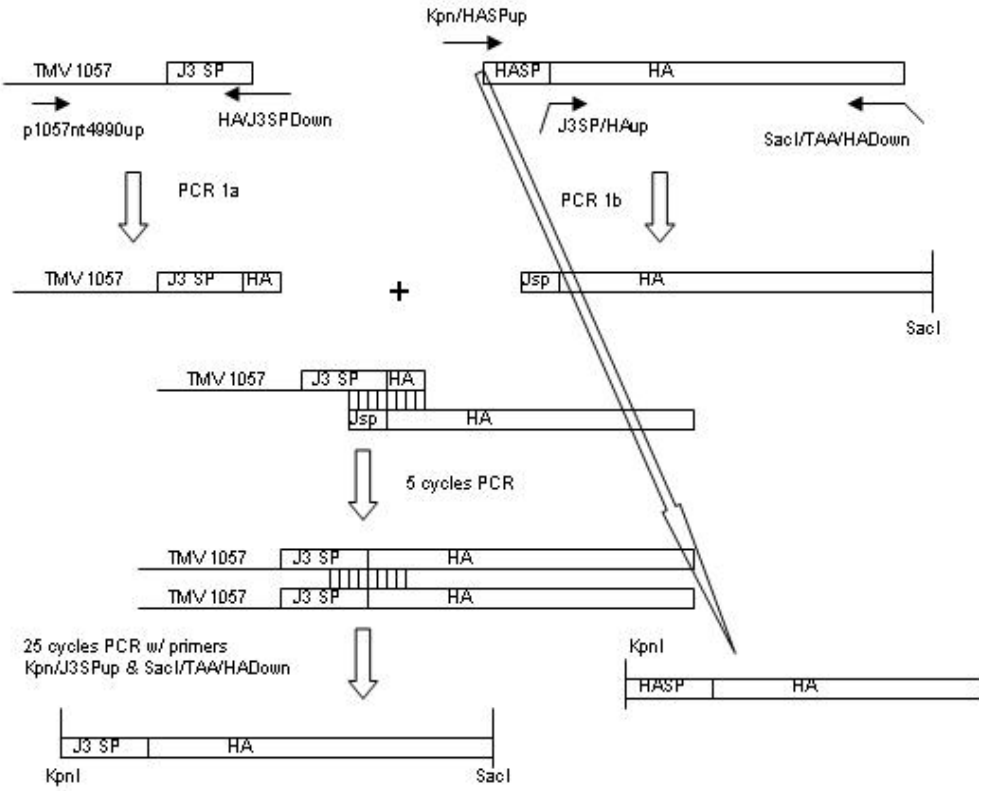

Figure 3. Schematic Depicting Construction Steps in Generation of HA3 Constructs. Construction of the two inserts was performed in several steps. The J3SP/HA3 insert was constructed by iterative PCR steps. The first PCR step, labeled PCR 1a, involved removing J3SP from TMV 1057 using primers p1057nt4990up and HA3/J3SPDown. This created a PCR product containing a portion of TMV 1057, the entire J3SP sequence, and a small portion of HA3. The second PCR step identified as PCR 1b, used the primers J3SP/HA3up and *SacI*/TAA/HA3down and added the end portion of J3SP, the entire HA3 sequence, and a *SacI* site at the 3' end. The PCR products from 1a and 1b were both used as template in one reaction for 5 cycles of PCR in order to extend the resultant product to encompass all of both templates. Finally, using PCR primers *KpnI*/J3SPup and *SacI*/TAA/HA3Down for 25 cycles of PCR yielded the first insert, *KpnI*/J3SP/HA3/*SacI*. The second insert, *KpnI*/HASP/HA3/*SacI*, was constructed using the PCR primers *Kpn*/HASPup and *SacI*/TAA/HA3Down. .

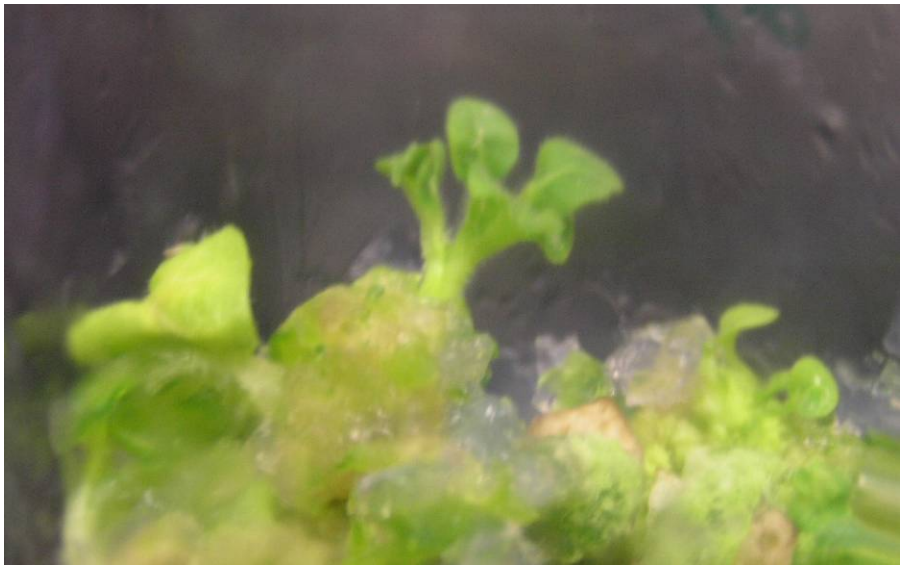

Figure 4. Close Up Picture of One Callus that is Developing a Transfected Plantlet in Antibiotic Agar Media. As detailed in the text, recombinant HA3 plasmids were transformed into *Agrobacterium tumefaciens*, and leaf fragments were exposed to the bacteria. Leaf fragments were cultured on antibiotic nutrient agar. Some fragments formed callus tissue, and some callus tissue formed new, transgenic plant shoots.

Four weeks later, surviving plants, which had formed calluses and then shoots (Fig. 4), were transferred to rooting medium and incubated for two weeks. Finally, they were transferred to soil (Fig. 5) (Biemelt et al. 2003, Horsch et al. 1985, Varsani et al. 2003). Roots are very sensitive to antibiotics, and the ability to root on medium containing antibiotics is a strong indication that transformation occurred (Draper et al. 1988).

To screen for HA3 expression, mRNA was extracted from leaves of fully grown plants (leaf size was at least 200mg). To examine HA3 mRNA expression, plant leaf mRNA extracts were prepared, using TRI Reagent (Ambion Inc, California, USA) and screened by reverse transcription-polymerase chain reaction (RT-PCR) (Huang et al. 2001, Lee and Suarez 2004, Marquet-Blouin et al. 2003, Payungporn et al. 2004, Satyavathi et al. 2003, Spackman et al. 2002, Varsani et al. 2003, Wong et al. 2006) for a three hundred base pair product from the 3' end of the HA3 ORF to include any cDNA

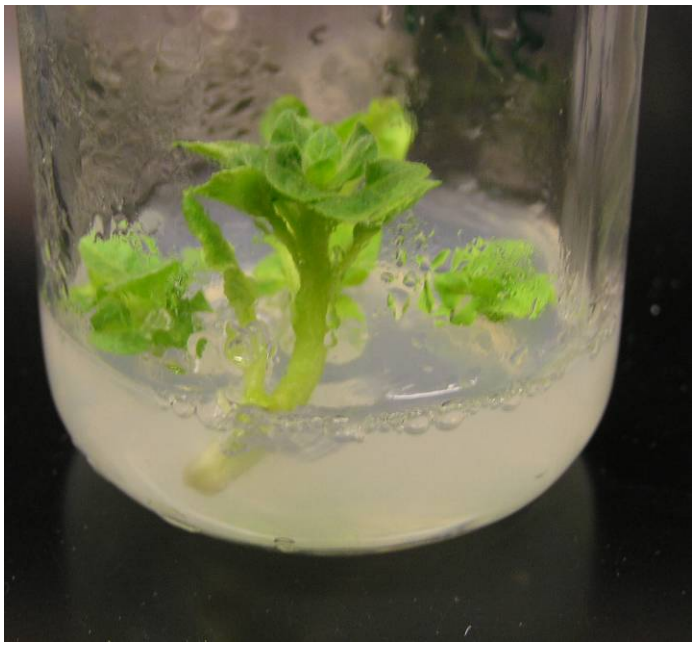

Figure 5. Candidate Transgenic Plant Growing on Rooting Agar Media. As described in the text, candidate shoots were transferred to rooting media and grown into plantlets. Plantlets were subsequently planted in soil and grown until adequately sized for leaf harvest.

products that may not have proceeded to the 5' end of the ORF. Screening utilized RT-PCR with 5' leader specific and 3' HA sequence specific primers. Specific primers were designed to form no primer dimers with a ΔG of -3 using DS Gene primer pair search (Accelrys, California, USA)and also checked with IDT website's OligoAnalyze (Integrated DNA Technologies, Iowa,USA). Using these specific primers in PCR screening for recombinant clones yielded a 300 base pair product. The HA3 screen forward primer sequence reads 5' AAGCACTCAAGCAGCCATC 3'. The HA3 screen reverse primer sequence reads 5' TGTCCTCAGCATTTTCCCTC 3'. PCR conditions consisted of an initial denaturation at 95^{o}C for 5 minutes, followed by 30 cycles at 95^{o}C for 1 minute, 57^{o}C for 1 minute, and 72^{o}C for 1 minute, terminating in a final elongation at 72^{o}C for 5 minutes. The PCR reaction for each sample contained 4μl of forward

primer, 4µl of reverse primer, 1µl of dNTPs (200µM each dNTP), 5µl of standard *Taq* buffer, 0.5µl of *Taq* polymerase (2.5U), 32.5µl of water, and 3µl of template DNA.

Since expression of this fragment of mRNA does not directly evaluate protein expression, clones positive for mRNA were subjected to immunoblot analysis to examine HA3 protein expression. From the highest expressing first generation HA3-*Nicotiana* transgenic plants, seed capsules were harvested and seeds collected, grown on agar plates for several weeks, and planted. From second generation plants, leaves were harvested, and interstitial fluid from the leaves of each plant was collected by vacuum infiltration. Extracts were concentrated by either PEG or ammonium sulfate precipitation and screened for HA3 expression by immunoblot (Howard and Streatfield 2003, Liu et al. 2005, Marquet-Blouin et al. 2003, Satyavathi et al. 2003, Varsani et al. 2003) to evaluate and verify HA3 transmissibility from generation to generation. Extracts were loaded and resolved through a 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel. Following electrophoresis the SDS-PAGE gel was electroblotted onto a polyvinylidene fluoride (PVDF) membrane. After blocking in 5% milk/phosphate buffer solution (PBS), the membrane was incubated in primary rabbit polyclonal anti-HA antibody-1 (Lab Vision, California, USA, Lot # 1438P511D) in PBS (1:10000). Following washing steps, the membrane was incubated in secondary goat anti-rabbit antibody conjugated to horseradish peroxidase (HRP) in PBS (Amersham, New Jersey, USA) (1:10000). Bands were visualized by chemiluminescence, using the Millipore Immobilon Western Kit (Millipore, Maryland, USA).

Construction of a Tobacco Mosaic Virus Vector Containing the HA3 cDNA

The previously cloned HA3 cDNA was sub-cloned, using PCR engineered *Avr* II sites on both ends of the ORF, as well as with an *Avr* II site at the 5' end of the HA3 ORF and a *Not* I site at the 3' end of the ORF in parallel. The *Avr* II/HA3/*Not* I HA3 product has the advantage of specific end sites, ensuring that clones generated from this PCR product can insert only in the sense direction for expression. This HA3 ORF was ligated into the TMV pJL36 vector (a gift from John Lindbo, Ohio State University) (Fig. 6), and DH10β electrocompetent cells were transformed with the resulting construct. Resultant kanamycin-resistant colonies were screened by colony PCR for HA3, using the same 3' primers as described previously. The anticipated potential problem of proper directional insertion of the *Avr* II/HA3/*Avr* II fragment, as opposed to the *Avr* II/HA3/*Not* I fragment, occurred and, because of the large number of *Avr* II/HA3/*Avr* II *E. coli* colonies that screened negative, the *Avr* II/HA3/*Avr* II construct was eventually abandoned in favor of the *Avr* II/HA3/*Not* I construct. Positive colonies were cultured, and plasmid mini-preps made for storage of the newly created vector.

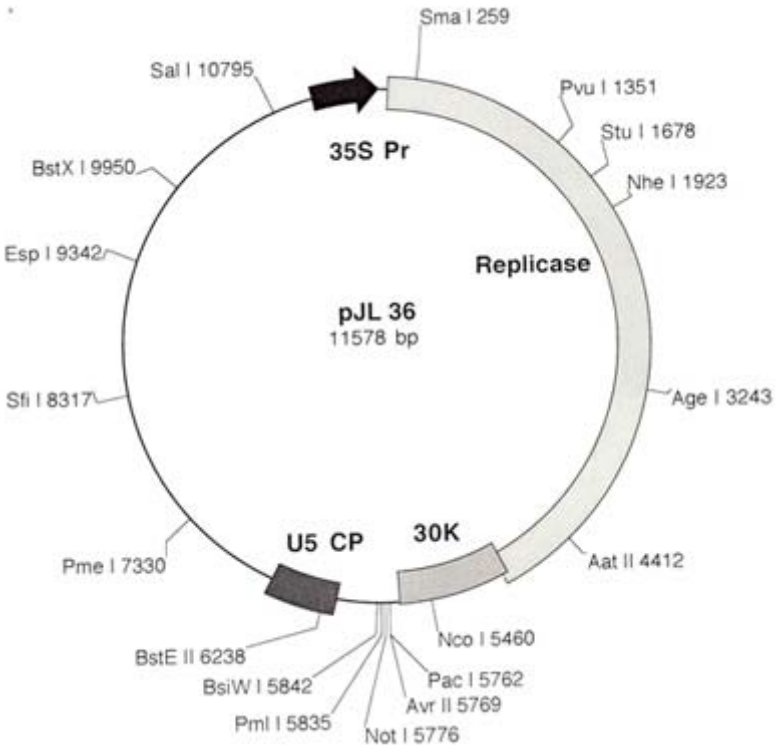

Figure 6. Binary TMV Vector pJL36. The vector contains all the essential TMV-U1 elements replicase, 30kDa movement protein (MP), and a TMV-U5 coat protein (CP). The multiple cloning site (MCS) was used for HA insertion. The 35S promoter allows for upregulation of the TMV RNA. Replicase codes for both a truncated protein (126 kDa) and a read-through version (183 kDa) which includes the RNA dependent RNA polymerase (Dawson and Lehto 1990). MP is required for cell-to-cell movement of viral RNA genomes by modifying plasmodesmatal junctions (Deom et al. 1990, Deom et al. 1992). CP is a structural protein required for encapsidation of the viral genome and systemic movement of the virus through the vascular system of the plant (Dawson et al. 1988). The viral genomic RNA codes for the two replicase proteins (Ishikawa et al. 1986) whereas the 30 kDa MP (Deom et al. 1987) and CP (Dawson et al. 1988) are coded for by subgenomic RNAs.

CHAPTER THREE

Results

Construction of a Functional Bacterial Plant Expression Vector with the Insert HA.

HA3 cDNA was isolated by PCR with sequence-specific primers, purified, and ligated into vector pE 1802 (Fig. 2). The subsequent construct was transformed into *E. coli*, and antibiotic resistant colonies were screened by PCR with HA3 specific primers (Fig. 7). Multiple positive clones were carried through subsequent steps.

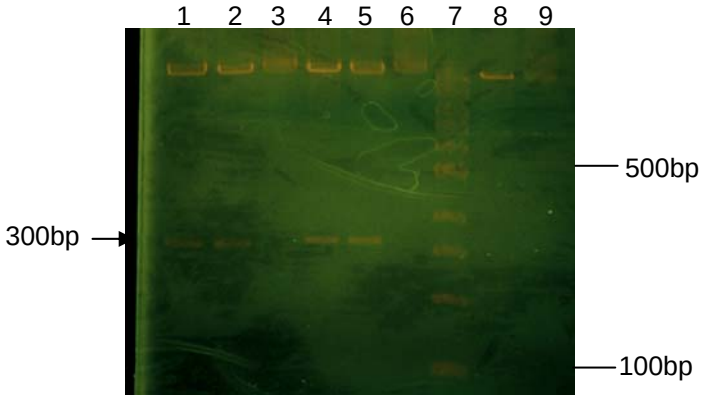

Figure 7. Positive Screen for HA3 Construct Clones. Following ligation of plasmid pE1802 with the HA construct inserts, DH5α bacteria were transformed and plated. Plasmid DNA from individual kanamycin-resistant colonies was purified and screened by restriction enzyme digest with *KpnI* and *SacI*. Successful recombination was indicated by a 300 bp drop-out band as seen in lanes 1, 2, 4, and 5. These constructs were carried forward in subsequent experiments. Lane 1, *KpnI*/HASP/HA3/*SacI*/pE1802 clone 1 cut with *KpnI* and *SacI*; lane 2, *KpnI*/HASP/HA3/*SacI*/pE1802 clone 2 cut with *KpnI* and *SacI*; lane 3, *KpnI*/HASP/HA3/*SacI*/pE1802 clone 1 uncut; lane 4, *KpnI*/J3SP/HA3/*SacI*/pE1802 clone 6 cut with *KpnI* and *SacI*; lane 5 *KpnI*/J3SP/HA3/*SacI*/pE1802 clone 7 cut with *KpnI* and *SacI*; lane 6, *KpnI*/J3SP/HA3/*SacI*/pE1802 clone 6 uncut; Lane 7, 1kb ladder (New England BioLabs); lane 8, pE1802 clone 1 cut with *KpnI* and *SacI*; lane 9 pE1802 clone 1 uncut.

Generation of Transgenic Plant Lines Expressing HA3 Protein

Following *Agrobacterium tumefaciens* based inoculation, culture on Murashige-Skoog medium and transfer to plant rooting medium, plantlets were transferred to soil and grown in the greenhouse. To examine HA3 mRNA expression, plant leaf extracts were screened by reverse transcription-polymerase chain reaction (RT-PCR). The RT-PCR products were subjected to agarose gel electrophoresis and evaluated for band presence and size (Fig. 8). The gel demonstrates that transgenic plant lines were generated which expressed HA3 mRNA transcripts.

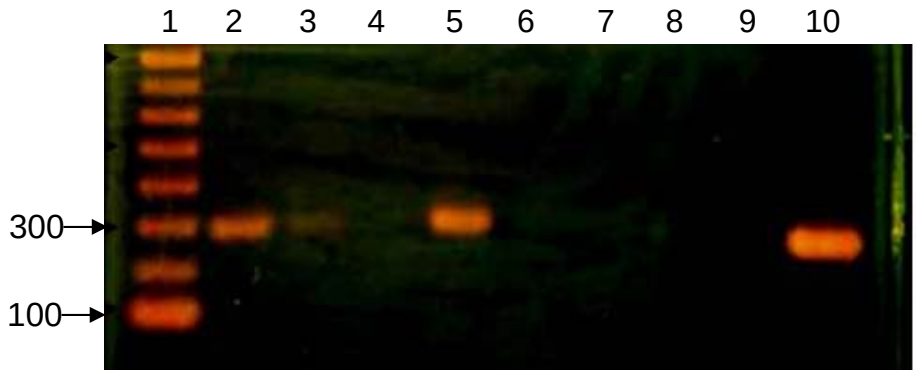

Figure 8. Agarose Gel of RT-PCR Products from HA3 Transgenic Tobacco Plants with Varying Leader Sequences. Lane 1 100-bp ladder (New England Biolabs), lane 2, J3SP HA3 (bacterial clone #6 sequence in *N. tabacum* plant line #2); lane 3, J3SP HA3 (clone #7 in *N. benthamiana* line #1); lane 4, HASP HA3 (clone #1 in *N. benthamiana* line #3); lane 5, HASP HA3 in *N. tabacum* line #1; lane 6 , the HA3 construct in a native *N. benthamiana* as a negative control, showing that no 300-bp product is produced from exists from the native *N. benthamiana* DNA genome. Lane 7 contains PCR products from the HA3 construct in native *N. tabacum* as a negative control, showing that no three-hundred base pair PCR product exists in the native *N. tabacum* DNA genome; lanes 8 and 9, *N. tabacum* and *N. benthamiana* transformed with just the parent vector pE1802 as negative controls. Lane 10, PCR of pUCIVHAsm, the source plasmid of the original HA sequence as a positive control.

Samples include extracts from plants containing the HA leader sequence or the Jun a 3 leader sequence. Extracts were made both from *N. Tabacum* and *N. benthamiana.* Two different bacterial clones were selected for each construction incorporating signal

peptides from HA (HASP) or Jun a 3 (J3SP). Several plant lines were screened representing of each of the four constructs and each of the two plant species. RT-PCR products in this gel are: lane 1 100-bp ladder (New England Biolabs, Massachusetts, USA), lane 2, J3SP HA3 (bacterial clone #6 sequence in *N. tabacum* plant line #2); lane 3, J3SP HA3 (clone #7 in *N. benthamiana* line #1); lane 4, HASP HA3 (clone #1 in *N. benthamiana* line #3); lane 5, HASP HA3 in *N. tabacum* line #1; lane 6 , the HA3 construct in a native *N. benthamiana* as a negative control, showing that no 300-bp product is produced from the native *N. benthamiana* DNA genome. Lane 7 contains RT-PCR products from the HA3 construct in native *N. tabacum* as a negative control, showing that no three-hundred base pair PCR product exists in the native *N. tabacum* DNA genome; lanes 8 and 9, *N. tabacum* and *N. benthamiana* transformed with just the parent vector pE1802 as negative controls. Lane 10, PCR of pUCIVHAsm, the source plasmid of the original HA3 sequence as a positive control.

These data are informative on several fronts. Amplification from our primer sets is robust as evidenced by the band from the HA3 plasmid positive control. Another conclusion is that the negative controls in lanes 6, 7, 8, & 9 verify the specificity of the primers; that is, a 300-bp product is not amplified with these primers from native genomes of *N. tabacum* or *N. benthamiana* nor from the unmodified vector, pE 1802. Finally, the figure shows that different plants with different vector constructs amplify HA3 cDNA to varying levels. This may indicate a "position effect" reflective of the tightness of histone binding at the point of gene insertion.

To identify continuous plant lines expressing HA3 protein, second generation plants were generated. Pods were harvested from the plants used for mRNA analysis,

and seeds were collected and planted. Protein extracts were made from resultant seedlings generated from the top mRNA expressing parental lines. These extracts were examined by immunoblot (Western blot) for HA3 protein expression (Fig. 9).

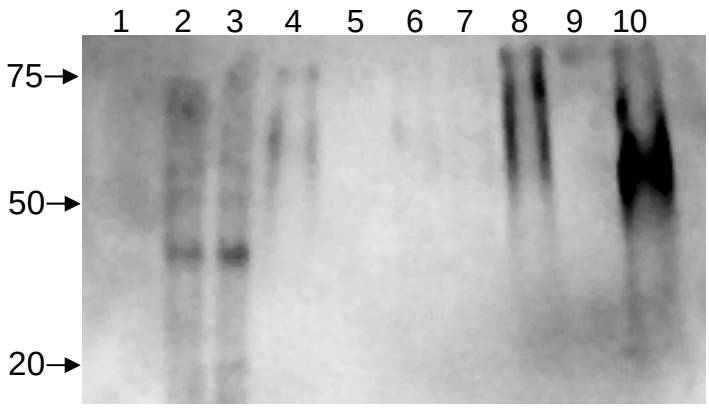

Figure 9. Immunoblot (Western blot) of Extracts from Infected Plants. Lane 1 contains the protein ladder (Full-range Rainbow Molecular Weight Markers, GE Healthcare). Lane 2 contains extract from wild type (nontransgenic) *C. elegans* (a gift from M. Lee, Baylor University) as a negative control. Lane 3 contains a positive control, extract containing an HA tagged protein extracted from *Caenorhabditis elegans* (a gift from M. Lee, Baylor University). Lanes 4-10 contain extracts from the test plants: lane 4,HASP1 sequence in *N. benthamiana* line 2; lane 5, J3 SP7 sequence in *N. tabacum* line 2; lane 6, J3SP6 sequence in *N. benthamiana* line 3 lane 7, HASP2 sequence in *N. tabacum* line 4; lane 8, HASP1 sequence in *N. benthamiana* line 4 lane 9, J3SP7 sequence in *N. tabacum* line 3; lane 10, J3SP6 sequence in *N. tabacum* line 3 .

Extracts were loaded in the following order: lane 1 contains the protein ladder (Full-range Rainbow Molecular Weight Markers, GE Healthcare). Lane 2 contains extract from wild type (nontransgenic) *C. elegans* (a gift from M. Lee, Baylor University) as a negative control. Lane 3 contains a positive control, containing an HA tagged protein extracted from *Caenorhabditis elegans* (gift from M. Lee, Baylor University). Lanes 4-10 contain extracts from the test plants: lane 4, HASP1 sequence in *N. benthamiana* line 2; lane 5, J3 SP7 sequence in *N. tabacum* line 2; lane 6, J3SP6 sequence in *N. benthamiana* line 3; lane 7, HASP2 sequence in *N. tabacum* line 4; lane 8,

HASP1 sequence in *N. benthamiana* line 4; lane 9, J3SP7 sequence in *N. tabacum* line 3; lane 10, J3SP6 sequence in *N. tabacum* line 3. The extracts were resolved through a 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel. Following electrophoresis the SDS-PAGE gel was electroblotted onto a polyvinylidene fluoride (PVDF) membrane. After the membrane was blocked in 5% milk/phosphate buffer solution (PBS), it was incubated in primary rabbit polyclonal anti-HA antibody-1 (Lab Vision, California, USA Lot # 1438P511D) in PBS. After washing steps, the membrane was incubated in secondary goat anti-rabbit antibody conjugated to horseradish peroxidase (HRP) in PBS (Amersham, New Jersey, USA). Bands were visualized by chemiluminescence, using the Millipore Immobilon Western Kit (Millipore, Maryland, USA).

From Fig. 9 we can draw some conclusions. First, the antibody appears to work accurately and specifically as evidenced by the band in lane 3, which represents the 20 kDa HA-tagged protein (*C. elegans* HA transgenic positive control) and is absent in lane 2 (*C. elegans* nontransgenic negative control). The expected MW of the soluble form of HA protein is 58 kDa, slightly larger if the signal peptide is intact. However, it has been previously reported that when expressed in *Pichia pastoris* and glycosylated, this same ORF has a MW of 78-85 kDa (Saelens et al. 1999). In Figure 9, we see a very large positive band, absent in the SDS-PAGE, which binds strongly to the HA antibody. This may be a glycosylated or oligomerized form of soluble HA3 (58 kDa).

Construction of a Tobacco Mosaic Virus Vector Containing the HA3 cDNA

The previously cloned HA3 cDNA was sub-cloned using a PCR engineered *Avr* II site at the 5' end of the HA3 ORF and a *Not* I site at the 3' end of the ORF. The HA3

ORF PCR product was ligated into the TMV pJL36 vector (a gift from John Lindbo, Ohio State University) (Fig. 6) and transformed into DH10β electrocompetent cells. Resultant kanamycin-resistant colonies were screened by colony PCR for HA3 using the same 3' primers as described previously (Fig 10).

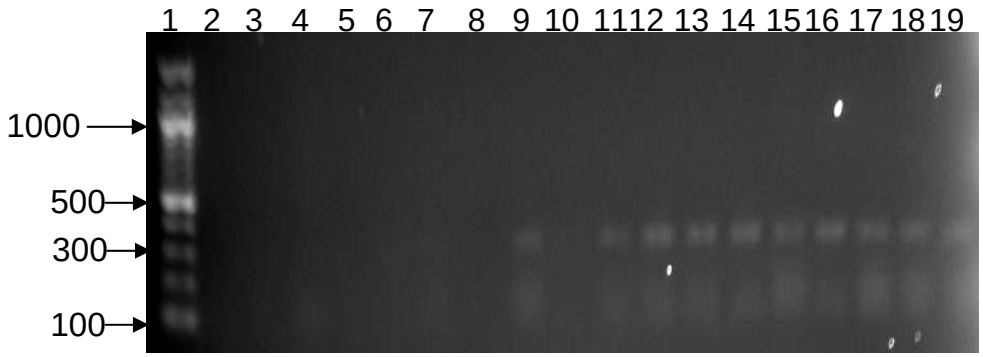

Figure 10. Agarose Gel of Colony PCR Products from *E. coli C*olonies**.** Lane 1, contains the 100-bp ladder (New England Biolabs), lanes 2-5, contain controls lanes of *E. coli* colonies transformed with the vector pJL36 without the insert of HA3 as negative controls. Lanes 6-19, contain the HA construct in pJL36 transformed into electrocompetent *E. coli* cells with the resulting colonies being screened. Positive results with a 300bp product, indicating that HA3 DNA is expressing, can be seen in lanes 9, 11, 12, 13, 14, 15, 16, 17, 18, 19.

The agarose gel in Figure 10 contains the products of the colony PCR screening for HA3 DNA in transformed *E. coli* colonies. Lane 1 contains the 100-bp ladder (New England Biolabs, Massachusetts, USA); lanes 2-5 contain controls lanes of *E. coli* colonies transformed with the vector pJL36 without the insert of HA3 as negative controls. Lanes 6-19 contain colony PCR products from the HA3 construct in pJL36 transformed into electrocompetent *E. coli* cells. Positive results with a 300bp product, indicating that HA3 DNA is present and replicating, can be seen in lanes 9, 11, 12, 13, 14, 15, 16, 17, 18, 19.

This gel is informative because it iterates that amplification from our primer sets is robust as evidenced by the bands for HA3 from the multiple lanes. Also, the negative controls in lanes 2, 3, 4, and 5 verify the specificity of the primers; that is, a 300-bp product is not amplified with these primers from *E. coli* colonies with only the pJL36 plasmid backbone. Finally, Figure 10 shows that some of the *E. coli* colonies are negative for pJL36/*Avr* II/HA3/*Not* I sequence, although most *E. coli* colonies yield PCR product for the insert pJL36/*Avr* II/HA3/*Not* I and that there are several positive clones to select from.

Several *E. coli* colonies were cultured and a plasmid mini-prep was performed from those cultures. The resulting pJL36 TMV vector with the HA3 insert cDNA was then saved. In the future this vector may be transformed into electocompetent *A. tumefaciens* cells (Biemelt et al. 2003, Huang et al. 2001, Liu et al. 2005, Marquet-Blouin et al. 2003) and then subepidermally injected into leaves of *N. benthamiana*, a technique known as agro-infiltration.

CHAPTER FOUR

Discussions and Conclusions

Hemagglutinin 3 (HA3) cDNA was isolated by PCR with sequence-specific primers and purified. The HA signal peptide or the Jun a 3 signal peptide was successfully attached to the 5' end of the ORF of HA3 cDNA. Then restriction enzyme sites *KpnI* at the 5' end of the HA3/ signal peptide ORF and *SacI* at the 3' end of the HA3 ORF were created by PCR addition. This construct, *KpnI*/signal peptide/HA3/*SacI,* was sub-cloned into the plant expression vector pE1802. Subsequently, the *KpnI*/signal peptide/HA3/*SacI/*pE1802 construct was transformed into DH5α *Escherichia coli* and plated on antibiotic selection plates. The resulting colonies were successfully screened by PCR for the presence of HA3 (Fig. 7). Several positive colonies were selected and transformed into *Agrobacterium tumefaciens.* The plasmid-transformed bacteria were introduced into *N. tabacum* as well as *N. benthamiana* by *A. tumefaciens* inoculation.

As the plants grew from 1-cm pieces to maturity, they were screened with antibiotics at several stages to confirm rentention of HA3 DNAAfter the plants reached the adult stage, mRNA was extracted from the plants, and an RT-PCR was performed. Gel analysis of the cDNA confirmed the presence of HA3 mRNA in both *N. benthamiana* and *N. tabacum* (Fig 8). From these positive HA3 mRNA-expressing plants, seeds were collected and cultured on antibiotic plates before being transplated to soil. From these second generation plants, protein was extracted and immunblot methodology was used to confirm that HA3 protein was expressing in the plants (Fig 9).

From these experiments some conclusions can be drawn. First, successful primer design and PCR parameters were established that enabled amplification of HA3 DNA, the addition of a signal peptide to the ORF of the HA3 DNA, and the addition of restriction enzyme sites to the end of the HA3 ORF/signal peptide. Second, the negative control samples in all three figures fail to yield a band at the desired location, indicating that no band in the region of the gel where HA3 bands are expected is present in native *N. benthamiana* and *N. tabacum.* Third, the inoculation approach employed appears to insert HA3 successfully into the genomes of *N. benthamiana* and *N. tabacum*. Fourth, the antibiotics used appear to successfully screen the tobacco plants by killing plants that did not take up the HA3 construct and allowing the plants that took up the HA3 construct to grow. Fifth, the antibody acquired appears to work accurately and specifically as evidenced by the band in lane 3, which represents the 20 kDa HA-tagged protein (*C. elegans* HA transgenic positive control) and which is absent in lane 2 (*C. elegans* nontransgenic negative control). Sixth, a very large HA3 positive band, absent in the SDS-PAGE, binds strongly to the HA antibody and at the expected molecular weight (Fig. 9). This indicates successful expression of HA3 in both *N. benthamiana* and *N. tabacum*.

HA3 was also successfully sub-cloned into TMV pJL36 vector. This occurred by PCR addition of *AvrII* and *NotI* restriction enzymes on the ends of the HA3 cDNA and then ligation of the *AvrII*/HA3/*NotI* insert and the TMV pJL36 vector. *E. coli* electrocompetent cells were transformed with the *AvrII*/HA3/*NotI*/pJL36 vector and a mini-prep was performed for storage of the cDNA. In future work, this vector will be transformed into *A. tumefaciens* electorcompetent cells and agro-inoculated into *N. benthamiana*.

The immunoblot from the transgenic *Nicotiana* plants (Fig. 9) shows an HA3 positive band at approximately 58 kDa. This HA3 band is comparable in size to the plant produced immunoblot band seen previously (Musiychuk et al. 2007) produced a band slightly larger than 60 Kda. The difference in band size is likely due to that fact that the previous work (Musiychuk et al. 2007) used HA5 (from the avain influenza H5N1) and linked it to stability protein lichenase. Also, previous work (Saelens et al. 1999) expressed HA3 protein in Pichia pastoris with a glycosyalted protein of 75 kDa and a deglycosylated protein of 58 kDa. The difference in size here is likely due to the fact that although glycosylation patterns of mammalian proteins expressed in transgenic plants are correct, different carbohydrate side chains are often used (Bardor et al. 1999). Complex glycans of plants are usually smaller than those of animals, since glycans expressed in plants lack sialic acid (Faye et al. 1993). The distortion of the HA3 band in Fig. 9, is due to the excessive amount of protein added into the lane; a similar distortion can be seen in the plant expressed HA5 protein (Musiychuk 2007). Additionally, variability in band sizes on SDS-PAGE may be a function of the experimental PAGE conditions used (Invitrogen, California, USA).

The range of HA3 mRNA expression levels seen among plant lines in this project is likely due to position effect. Position effect is the variance in gene expression level dependent on the transgene's location within the genome compared to that of other transgene constructs(Weiler and Wakimoto 1995). Although little is known about the nature of position effect, its existence is widely acknowledged (Clark et al. 1994). Position effects are not controllable, and this means that the frequency and level of transgenic expression is not predictable and must be empirically examined (Clark et al.

1989, Clark et al. 1994, Whitelaw et al. 1991). Due to position effects, very little correlative relationships can be seen between the number of constructs inserted into the genome and the level of transgenic expression (Archibald et al. 1990). Because of position effects, some regions of the genome are more permissive for transgenic expression than others (Clark et al. 1994). This position effect is most likely the explanation for the fact that some plants express the HA3 mRNA is greater quantities than other plants, and thereby why some plants express HA3 protein in greater quantities than other plants.

The HA3 expressed in transgenic plants in this project has several advantages over the HA3 produced in *P. Pastoris*. HA3 expressed in transgenic plants has the advantage of correct post-translational modifications, since transgenic plants produce protein with post-translational modifications similar to those of higher eukaryotes (Streatfield and Howard 2003) that *P. pastoris* does not. HA3 produced in transgenic plants is cheaper than HA3 produced in *P. pastoris* because *P. pastoris* protein expression requires expensive technical equipment and facilities (Baneyx 1999, Butler et al. 2003, Hannig and Makrides 1998, Harashima 1994, Hunt 2005, Makrides 1996, Palomares et al. 2004, Roodveldt et al. 1995, Streatfield and Howard 2003, Varsani et al. 2003). Also, once functional HA3 has been expressed by TMV it will have the advantage of reduced production time compared to HA3 expressed in *P. pastoris*. However, the HA3 protein expressed in transgenic plants lacks the lichenase stability protein that the HA5 protein has. Thus the HA3 protein expressed in transgenic plants has the disadvantage of reduced production yield compared to the HA5 protein, and not surprisingly, the HA5 protein is therefore more readily purified than the HA3 protein.

All future work may benefit from the AvrII/HA3/NotI/pJL36 vector to agro-inoculate *N. benthamiana*. The TMV vector has a time advantage over transgenic plants for the HA3 protein expression; one week after agro-inoculation, the HA3 protein is ready for extraction (Kumagai et al. 2000, Musiychuk et al. 2007). In contrast it takes many months before the HA3 protein is ready for extraction from transgenic plants. Additionally, TMV should be used instead of transgenic plants because plant virus vectors allow for easy purification of the inserted proteins from the host plant (Yusibov et al. 1999). Moreover, since there is no natural vector to transmit TMV into crop plants, it is less biohazardous than other vectors and than transgenic plants (Pogue 1998).

The overall specific aim of the HA3 construction and protein expression experiments is to evaluate the ability of plant-expressed influenza HA3 to function as an immunogen *in vivo* to generate protective antibodies against an influenza challenge. These experiments will eventually be conducted by a collaborator in a laboratory off site. Folllowing transformation of the *AvrII*/HA3/*NotI*/pJL36 vector into *A. tumefaciens* electrocompetent cells, the cells will then be cultured for three days. The cells will then be placed into induction buffer and agro-inoculated into *N. benthamiana* plants. After a one week incubation, the plants will be evaluated for HA3 protein expression (Musiychuk et al. 2007). The best HA3 protein expression plant(s) will be expanded to produce a sufficient amount of HA3 protein to be collected, purified, and evaluated for use in immunological challenge experiments. The purified HA3 protein will be sent to a collaborator (David Woodland, Trudeau Institute, NY) for use as an influenza challenge in mice to examine whether HA3 protein expressed in plants has a native fold and whether the secreted HA3 protein can elicit an immunological response. If the HA3

protein does not elicit an immune response, or if the immune response (i.e., anti-HA antibodies) does not block or subvert the influenza challenge infection, it may mean that one or more important epitopes are blocked or inappropriately modified for those functions. All of these experiments will follow the appropriate NIH rules and regulations for animal experiments and will be approved by the institutional animal use and care committee (IACUC) of that institution. Once this future work is successful it will benefit all individuals seeking an influenza vaccine, because influenza vaccine production will be greatly enhanced and production of this vaccine will be less expensive. Hopefully this will allow all individuals to obtain this necessary vaccine every year.

APPENDIX

Benchtop Protocols

Chemicals

Unless otherwise stated, laboratory chemicals were purchased from VWR International, Merck, Sigma, or Fisher and were of the analytical or the highest grade available. All restriction enzymes were bought from NEB. In vitro transcription kits were purchased from Ambion Inc.

Media, Stock Solutions, and Buffers

Standard Media and buffers were prepared according to standard procedures using deionized water and sterilized by autoclaving (15 min/121ºC/15 psi). Heat-sensitive components, such as antibiotics, were prepared as stock solutions, filter-sterilized (0.2µm) and added to the medium/buffer after cooling to 50ºC. If a percentage is given for a component in media or buffer recipes (e.g. 10% NaCl), it describes a weight per volume (w/v) ratio, unless otherwise indicated.

Protocols for DNA, RNA, and protein experiments are listed separately

DNA Protocols

Plasmid Mini-Preps (Current Protocols)

Alternatively, any plasmid mini-prep kit with a spin column (e.g. Sigma-Elute or Qiagen) may be used conveniently following the instructions from the company.

1. Grow an overnight culture in 5ml LB/ampicillin or TB/ampicillin in a 25-ml glass culture tube.

2. Spin down 1.5ml of culture in a microcentrifuge tube at high speed for 15 seconds to get a soft pellet. Decant and aspirate supernatant. Repeat with an additional 1.5ml of overnight culture. For large scale plasmids repeat with a third 1.5ml.
3. Add 100µl GTE. Vortex or pipette to resuspend cells.

4. Add 200µl freshly made 0.2 NaOH/1% SDS. Mix well by gently inverting. Keep on ice for 5 min.

5. Add 150µl 3M Kac (pH 4.8). Mix well by gently inverting. Keep on ice for 5 min.

6. Microcentrifuge at 4ºC for 10 min. Transfer supernatant to a new tube.

7. Add 20ul 1mg/ml RNase A. Incubate at 37^{o}C for 20 min.

8. Extract twice with equal volume of 1:1 phenol:chloroform and once with chloroform. Vortex vigorously for 10 seconds and spin for 15 seconds at room temperature.

9. Add 2.5x volume of 100% ethanol. Place in -20^{o}C freezer for 15 min. or longer. Microcentrifuge at high speed for 12 min. Perform 70% ethanol rinse by applying equal volume of 70% ethanol, spin for 5-12 min., pipette off liquid, and vacuum dry for 7min. or until dry. Resuspend in 20µl TE and store at -20^{o}C.

Plasmid Maxi-prep (Current Protocols)

1. Inoculate one ampicillin/TTC plate with *E. coli* containing vector. Incubate overnight.

2. Pick smallest colony and inoculate 5ml TB. Grow overnight. (5ml TB and 40µl stock ampicillin) and incubate at 37^{o}C shaker at 300 rpm.

3. Inoculate two 250ml TB (flasks) with this culture. 2.5ml to each flask. Grow until saturated or an O.D. 600 of approximately 6.

4. Harvest the bacterial cells from 500µl culture by centrifugation at 6000 x g for 10 min. at 4^{o}C. Invert tube for 15 min. to let them dry completely.

5. Resuspend pellet with pipettor by adding 2ml GTE solution in each tube and pour suspensions in single tube.

6. Add 1ml 25mg/ml hen egg white lysozyme (made fresh in TE). Resuspend the pellet completely in this solution and allow to stand 20 min. at room temperature.

7. Add 10ml freshly prepared 0.2 M NaOH/ 1% SDS and mix by stirring gently with a pipette. Let stand on ice for 10 min.

8. Add 7.5ml, 3 M KAc, stir for 5 min., viscosity reduces and large floating precipitate forms. Let stand 10 min. on ice.

9. Centrifuge for 10 min. at 10000 x g in JA 25.5 at 4ºC.

10. Filter with cheesecloth and add 6ml isopropanol.

11. Centrifuge at room temperature for 10 min. at 10000 x g. Wash pellet with 2ml cold ethanol, centrifuge briefly to collect pellet, aspirate off ethanol.

12. Dry pellet under vacuum and dissolve in 2ml TE.

13. Divide 2ml plasmid between four eppendorf tubes. Add 1µl (10 mg/ml) RNase to each of them and incubate 37^{o}C for 20 min. Chloroform extract plasmid twice or thrice, depending on the amounts of protein seen on the interface. Hand warm RNase prior to use.

14. Add one-fourth volume of 10 M ammonium acetate.

15. Add two volumes of ice cold 100% ethanol.

16. Incubate in -20^{o}C for 60 min.

17. Centrifuge in JA 25.5, 17000 x g for 10 min.

18. Pour off supernatant, wash pellet with 2ml 70% ethanol and vacuum dry.

19. Resuspend pellet in 2ml TE buffer, add 0.8ml PEG/NaCl, and incubate at 4^{o}C. Centrifuge PEG precipitate at 4^{o}C for 20 min.

20. Resuspend pellet in 1ml TE buffer and vortex.

21. Add 100ml 3 M sodium acetate, pH 5.3, mix by vortexing briefly.

22. Add 2.2ml ice cold 100% ethanol, vortex, incubate at -20^{o}C.

23. Centrifuge in JA 25.5 rotor at 10000 x g for 20 min at 4^{o}C. Pour off supernatant.

24. Add 600µl ethanol. Centrifuge, dry under vacuum.

25. Add 100µl deionized water and quantify by spectrophotometer and fluorometer. Check the purity of plasmid DNA by running on agarose gel. Store in small aliquots in -20^{o}C.

Bacterial Transformation (Electroporation) (ECM 600 by BTX)

1. Check to see if appropriate plates available for the transformants; if not, make then first.

2. Thaw SOC tube in 37^{o}C bath.

3. Gently thaw competent cells on ice. Label and place clean (sterile) 1mm-gap cuvettes on ice, along with labels 1.5ml microfuge tubes.

4. Look up correct electroporation settings in BTX manual and set machine (protocol 013 is for DH5ά *E. coli*).

5. Set up the following controls: 1) no insert (uncut vector) control to check minipreps without digestions, 2) 10pg of pUC to check transformation efficiency, and 3) self-ligated control (no insert) to check background.

6. Add 40µl of competent cells to microfuge tubes. (Cells should be out of ice only for a moment. Lift up tubes and transfer with pipetor quickly.)

7. Add 1µl of DNA (dilute ligation mix 1:5 in water first) to microfuge tubes and mix be pipetting only once or twice.

8. Transfer to cuvettes. The cells will stay towards the top of the gap.

9. Quickly wipe off ice and put cuvette into holder firmly. Close top.

10. Press the pulse button, When light stops blinking, the charge has been delivered. Note voltage and msec duration. This should be close to the initial value displayed.

11. Remove cuvette and add 1ml SOC (should be room temperature). Replace cuvette cap, pipet in and out a few times very gently; pipet into new labeled sterile tube.

12. Press reset on the electroporator and repeat for other samples.

13. Place samples in 37^{o}C incubator for 1 hour. If desired may add a little bit of glucose to samples to help cells recover from electroporation. Also, if desired sample may be shook during incubation hour.

14. After 1 hour, plate out samples in predetermined volumes. The more competent the cells, the less sample needed for plating. The weaker the ligation, the more sample needed for plating.

15. Spread culture over media's plate surface. Incubate plates upside down in 37°C incubator overnight.

Bacterial Transformation (Chemical)

1. Thaw competent cells on ice. Place required number of sterile tubes on ice.

2. Chill approximately 5ng (2µl) of the ligation mixture in a 1.5ml microcentrifuge tube.

3. Add 50µl of competent cells to the DNA and mix gently by pipetting up and down.

4. Incubate on ice for 30 min.

5. Heat shock for 45 seconds at 42°C, chill on ice for 2 min.

6. Add 950µl of room temperature SOC or LB media and incubate at 37°C for 1 hour. Also, if desired, may add a little bit of glucose to help the cells recover.

7. Shake at 225 rpm (37°C) for 1 hour.

8. Spread onto the appropriate number of plates. Amount to be spread depends on the competency and the ligation strength. If more competent cells used, less volume should be spread. If a weak ligation occurred, then more volume should be spread.

9. Incubate plates upside down overnight at 37°C.

Solutions and Buffers

- SOC Media

1. For 100ml media, weigh 2.0g bacto-tryptone, 0.5g bacto-yeast extract, and 0.05g NaCl.

2. Dissolve in an appropriate volume of dH_2O.

3. Add 10ml of a 250mM solution of KCl. (1.86g KCl in 100ml of dH_2O.)

4. Adjust pH to 7.0 with 5N NaOH. Bring up to final volume and autoclave.

5. Allow to cool to 60°C or less and then add 20ml sterile 1M glucose. (Dissolve 18g glucose in 90ml ddH_2O. After the sugar has dissolved adjust the volume to 100ml with ddH_2O.)

6. Just before use, add 5ml of a sterile 2M $MgCl_2$ solution. (Dissolve 19g of $MgCl_2$ in 90ml of ddH_2O and adjust the volume to 100ml with ddH_2O and sterilize.)

RNA Protocols

RNA Extraction from Plants (Molecular Research Center) (TRI Reagent®)

1. Homogenize plant leaves in TRI Reagent (1ml/50-100mg plant leaves) using chilled mortar and pestle. Sample volume should not exceed 10% of the volume of TRI Reagent used for homogenization. Avoid washing cells before the addition of TRI Reagent as this may contribute to mRNA degradation.

2. Store the homogenate for 5 min. at room temperature to permit complete dissociation of nucleoprotein complexes.

3. Add 0.1ml BCP (bromochloropropane) or 0.2ml chloroform per 1ml of TRI Reagent, cover samples tightly and shake vigorously for 15 seconds.

4. Store the resulting mixture at room temperature for 2-15 min. and centrifuge at 12000 x g for 15 min. at 4°C. Following centrifugation, the mixture separates into a lower red phenol-chloroform phase, interphase, and organic phase. The volume of the aqueous phase is about 60% of the volume of TRI Reagent used for homogenization. BCP is less toxic than chloroform and its use reduces the possibility of contaminating RNA with DNA. Chloroform used for phase separation should not contain isoamyl alcohol or any other additive. It is important to perform centrifugation to separate aqueous and organic phases in the cold (4-10°C). If performed at elevated temperatures, a residual amount of DNA may sequester in the aqueous phase. In the case, RNA can be used for northern analysis but it may not be suitable for PCR.

5. Transfer the aqueous phase to a fresh tube and save the interphase at 4°C for subsequent isolation of DNA and proteins. Precipitate RNA from the aqueous phase by mixing with isopropanol (0.5ml isopropanol per 1ml of TRI Reagent used in initial homogenization).

6. Store samples at room temperature for 5-10 min. and centrifuge at 12000 x g for 8 min. at 4-25°C. RNA precipitate (often invisible before centrifugation) forms a gel-like or white pellet on the side and bottom of the tube. One may have trouble isolating RNA from sources rich in polysaccharides and proteoglycans.
7. Remove the supernatant and wash the RNA pellet (by vortexing) with 75% ethanol and centrifuge at 7500 x g for 5 min. at 4-25°C. Add at least 1ml of 75% ethanol per 1ml TRI Reagent used for initial homogenization. If the RNA pellet

accumulates on the side of the tube and has a tendency to float, sediment the pellet at 12000 x g.

8. Remove the ethanol wash and briefly air dry the RNA pellet for 3-5 min. It is important not to completely dry the RNA pellet as this will greatly decrease its solubility. Do not dry RNA by centrifugation under vacuum. Drying is not necessary for solubilization of RNA in FORMazol® (a form of stabilized formamide). Dissolve RNA in FORMazol®, water, or 0.5% SDS by passing the solution a few times through a pipette tip and incubating for 10-15 min. at 55-60^{o}C. Water or the SDS solution used for RNA solubilization should be made RNase-free by diethyl pyrocarbonate (DEPC) treatment. RNA should be precipitated from FORMazol® with ethanol before using for RT-PCR.

1st Strand cDNA Synthesis (*Reverse Transcription PCR*) (Promega)

1. In a sterile RNase-free microcentrifuge tube add 2µg RNA template.

2. Add primer at a concentration of 0.5µg per µg of RNA sample in a total of 10µl in sterile ddH_20. The ratio of primer to mRNA should not be altered.

3. Heat the tube at 70^{o}C for 5 min. to melt secondary structure within the template and then place on ice to prevent secondary structure from reforming.

4. Centrifuge the tube briefly to collect the solution at the bottom of the tube and add the master mix to the annealed primer/template.

5. The master mix (1X) should consist of the following:

M-MLV Reaction buffer (5X, Promega)	5µl
dNTPs (10mM)	5µl
rRNasin Ribonuclease Inhibitor, 25U (Promega, 40 U/µl)	4µl
M-MLV Reverse Transcriptase, 200U (Promega, 200U/µl)	0.625µl
Sterile deionized water to final reaction volume of 25µl	3.375µl

6. Mix gently by flicking the tube and incubate for 60 min. at 37^{o}C for random primers or 42^{o}C for other primers or primer-adaptors.

7. Heat-inactivate the reverse transcriptase by incubating at 70^{o}C for 15 min. The following addendum is utilized for Real-Time PCR.

8. The cDNA can now be used for amplification in PCR. However, amplification of PCR targets for real-time PCR requires the removal of unreacted RNA. Extraneous RNA can be removed by adding 1U of E. Coli RNase H (Promega, 1.5U/µl) and incubating for 20 min. at 37^{o}C.

9. The 1st strand cDNA can be stored at -20^{o}C until further use.

Protein Protocols

Protein Extraction by Vacuum Infiltration (McCormick et al. 1999; McCormick et al. 2003)

This protocol will extract proteins that are secreted to the interstitial fluid of the plant

1. In a 100ml beaker, combine 2g of leaf material and 50ml of Infiltration Buffer. Ensure leaves are completely covered in buffer.

2. Place beaker into a vacuum chamber. Apply vacuum for 2 min. Remove vacuum by allowing air to flow into chamber in one sudden single burst. Leaves should turn darker in color and sink.

3. Remove the leaves from the beaker and gently dry with paper towels. Place the leaves into a strainer cup that has been positioned into a 250ml centrifuge tube.

4. Centrifuge at 2000 x g for 10 min. at 4^{o}C. Secreted protein will be in the bottom of the centrifuge tube.

5. Should make Infiltration Buffer fresh from stocks and store no more than a few days at 4^{o}C.

Protein Extraction by Grinding

This protocol can be used on either whole leaves or to extract non-secreted protein from leaves previously used in Vacuum Infiltration protocol.

1. Freeze leaves in a chilled mortar-pestle using liquid nitrogen.

2. Immediately after the liquid nitrogen has evaporated, grind 200mg of the frozen leaves using 500µl Extraction Buffer. Transfer liquid to a microcentrifuge tube.

3. Centrifuge at 13000 x g for 30 min at 4^{o}C.

4. Transfer supernatant to a new tube.

5. An additional optional step, centrifuge for 20 min at 13000 x g at 4^{o}C and transfer to a new tube.

6. It is better to make fresh extraction buffer from stocks and store no more than a few days at 4^{o}C.

Making an SDS-PAGE Gel

1. Assemble gel plates with spacers according to the manufacturer's instructions. Gel spacers can either be 0.75mm or 1.5mm in width.

2. If the gel apparatus does not have a plug for the bottom, then warm up some 1% agarose and place it in the bottom of the gel plate.

3. Mark the level to which the separating gel should be poured, approximately a few mm below the level where the wells will be formed by the comb.

4. Determine what percent gel should be made based on the size of the protein looking for. The larger the protein, the less percent gel should be made. The smaller the protein, the greater the percent gel should be made.

5. For the resolving gel, to an appropriate volume tube first add ddH_2O:

mls of gel	5	10	15	20
% gel 8	2.61ml	5.22ml	7.84ml	10.45ml
10	2.28ml	4.56ml	6.84ml	9.11ml
12	1.95ml	3.89ml	5.84ml	7.78ml
15	1.45ml	2.89ml	4.34ml	5.78ml

6. Next add the 5X Resolving gel buffer:

mls of gel	5	10	15	20
	1ml	2ml	3ml	4ml

7. Next add the 30% acryl/bis:

mls of gel	5	10	15	20
% gel 8	1.33ml	2.67ml	4.00ml	5.33ml
10	1.67ml	3.33ml	5.00ml	6.67ml
12	2.00ml	4.00ml	6.00ml	8.00ml
15	2.50ml	5.00ml	7.50ml	10.00ml

8. Before adding the 10%APS and the TEMED, go ahead and set up your pipettes with the proper amounts and put tips on them. Also, set up a 1ml pipette with 1ml and place a tip of it. This should be done before adding these reagents because once these reagents are added to the gel, the gel will start to polymerize quickly.

9. After that, add the 10% APS:

mls of gel	5	10	15	20
	0.05ml	0.1ml	0.15ml	0.2ml

10. Finally add the TEMED:

mls of gel	5	10	15	20
	0.005ml	0.01ml	0.015ml	0.02ml

11. Once the TEMED has been added quickly mix the tube gently by inverting a few times and then pour the gel into the already preassembled gel plates.

12. Wait for the gel to start to polymerize, then add a thin layer off water saturated butanol to the top of the resolving buffer, this will help to make the gel have a straight top and also to remove any air bubbles from forming in the resolving buffer.

13. Once the resolving gel has fully polymerized, remove the water saturated butanol.

14. Next, make the stacking gel, to an appropriate volume tube first add ddH_2O:

mls of gel	1	2	3	4
	0.72ml	1.44ml	2.17ml	2.89ml

15. Then, add the 10X stacking gel buffer:

mls of gel	1	2	3	4
	0.10ml	0.20ml	0.30ml	0.40ml

16. After that, add the 30% acryl/bis:

mls of gel	1	2	3	4
	0.17ml	0.33ml	0.50ml	0.67ml

17. Before adding the 10%APS and the TEMED, go ahead and set up your pipettes with the proper amounts and put tips on them. Also, set up a 1ml pipette with 1ml and place a tip of it. This should be done before adding these reagents because once these reagents are added to the gel, the gel will start to polymerize quickly.

18. Next, add the 10%APS:

mls of gel	1	2	3	4
	0.01ml	0.02ml	0.03ml	0.04ml

19. Finally, add the TEMED:

mls of gel	1	2	3	4
	0.001ml	0.002ml	0.003ml	0.004ml

20. Once the TEMED has been added quickly mix the tube gently by inverting a few times and then pour the gel into the already preassembled gel plates on top of the resolving gel. Then, add the combs to form the wells, make sure that no air bubbles are present next to or under the wells.

Running an SDS-PAGE gel

1. After the stacking gel has fully polymerized, the gel apparatus can be placed in the electrophoresis chamber. (Alternatively, the gel can be saved at least for a couple of days at 4^oC in ddH_2O or running buffer.) Fill the chamber with 1X

electrophoresis buffer (running buffer), remove the comb, and wash the wells with running buffer before loading samples.

2. Prepare the samples for electrophoresis by adding the appropriate amount of either 2X of 5X loading dye, boiling for 10-15 min., performing a quick spin, and then loading in the wells.

3. Run gel(s) at 45mA until the loading dye reaches the bottom of the gel.

Transfer of Protein from Gel to PVDF Membrane (Biorad Trans-Blot® SD Semi-Dry Electrophoretic Transfer Cell; 170-3940)

1. Prepare the transfer buffer while the SDS-PAGE gel is running.

2. Equilibrate the gels in transfer buffer. 15 min for a 0.75mm SDS-PAGE and 30min for a 1.5mm SDS-PAGE. To prevent diffusion of low molecular weight proteins, change pre-equilibrium buffer several times during a short pre-equilibrium period.

3. Cut the PVDF membrane to the dimensions of the gel. Wet the membrane by slowly sliding it at a 45 degree angle into transfer buffer and allow soak for 15-30 min. Completely wetting the membrane is important to ensure protein binding.

4. Cut filter paper to the dimensions of the gel. Two pieces of extra thick filter paper or four pieces of thick filter paper. Completely saturate the filter paper by soaking in transfer buffer.

5. Remove the safety cover and the stainless steel cathode assembly. (Remove the white plastic border also.)

6. Place a pre-soaked sheet of extra thick filter paper onto the platinum anode. Roll a pipette or test tube over the surface of the filter paper to exclude all air bubbles.

7. Place the pre-wetted membrane on top of the filter paper. Roll out all air bubbles.

8. Carefully place the equilibrated gel on top of the transfer membrane, aligning the gel on the center of the membrane. Roll out any air bubbles.

9. Place the other sheet of pre-soaked filter paper on top of the gel, carefully removing any air bubbles from between the gel and filter paper.

10. Carefully place the cathode onto the stack. Press to engage the latches with the guide posts without disturbing the filter paper stack.

11. Place the safety cover on the unit. Plug the unit into the power supply. Normal transfer polarity in cathode to anode. Don't reverse the polarity.

12. Turn on the power supply. Transfer mini gels for 15-30 min. at 10-15V. Following transfer, turn off power supply, and disconnect the unit from the power supply. Remove the safety cover and the cathode assembly. Discard filter paper.

Transfer of Protein from Gel to PVDF Membrane (Wet Transfer)

1. Prepare the transfer buffer while the SDS-PAGE gel is running and place at 4^{o}C.

2. Also, prepare the PVDF membrane for transfer. Cut the PVDF membrane to the appropriate size and soak in methanol. Once, the membrane is saturated transfer the membrane into water. Do not touch the membrane with your hands, or use membrane forceps to move a PVDF membrane.

3. Once the SDS-PAGE gel has finished running, take the gel/membrane holder and place in a dish, remembering that the black side goes on the bottom.

4. Remove the transfer buffer from the 4^{o}C and pour some into the dish with the gel/membrane holder. Wet a sponge in transfer buffer and place on the black side of the gel/membrane holder. Then wet three pieces of filter paper on top of the sponge. Use a pipette or test tube to remove any air bubbles.

5. Remove the gel from the gel plates and remove the stacking gel and the agarose plug. Then place the gel on top of the filter paper.

6. Take the PVDF membrane and place it directly over the gel, so that the gel is entirely covered by the PVDF membrane. Remove any air bubbles by rolling a test tube or pipette over the top of the PVDF membrane.

7. Wet three more pieces of filter paper with the transfer buffer and place on top of the PVDF membrane, removing any air bubbles.
8. Finally, wet another piece of sponge with transfer buffer and place on top of the filter paper. Then clamp the top of the gel/membrane holder shut being careful not to disturb its contents.

9. Then place the gel/membrane holder into the transfer box. Pour any excess transfer buffer from the dish where the gel/membrane holder was assembled into the transfer box. Fill the transfer box with the remaining transfer buffer made. Add a stir bar into the bottom of the box. A stir bar big enough to circulate the transfer buffer, but that does not touch the gel/membrane holder.

10. Place the transfer box into of a dish full of ice (to keep the transfer buffer cold) (make sure the dish is no taller than one-fourth the height of the transfer box so

that ice or cold water will run into the transfer box diluting the transfer buffer). Also, have the dish full of ice sitting on top of a stir plate so the stir bar will stir the transfer buffer.

11. Set the power supply to run at 300mA for 55 min. (for a 0.75mm gel) or for 1 hour 15 min. (for a 1.5mm gel).

Western Analysis of Protein on a PVDF Membrane

1. After transferring proteins to PVDF membrane, block the membrane for 30 min/1 hour/overnight in 5% milk in desired buffer (PBS,PBS-T, TBS, TBS-T). The blocking buffer needs to be made fresh right before use.

2. Rinse the membrane thrice in desired buffer and then wash the membrane in desired buffer thrice for 5 min. each.

3. Incubate membrane in primary antibody (1:5000 dilution or 1:10000 dilution) at room temperature for 1 hour with gentle agitation (rocking of shaking).

4. Rinse the membrane thrice in desired buffer and then wash the membrane in desired buffer thrice for 5 min. each.

5. Incubate membrane in secondary antibody (horseradish peroxidase linked antibody) (1:1000 dilution, 1:2000 dilution, or 1:5000 dilution) at room temperature for 1 hour with gentle agitation (rocking or shaking).

6. Rinse membrane thrice with desired buffer and then wash the membrane in desired buffer thrice for 5 min. each.

7. Develop membrane with 2ml of enhanced chemiluminescence (ECL) reagent.

Silver Staining of Western Membranes-works with both PVDF and Nitrocellulose

1. Add membrane and staining solution together in a container just larger than the membrane and shake, rock, or gently agitate until light bands develop.

2. Drain the stain solution off and immediately add as large a volume of fresh ddH_2O as possible. Rotate to rinse briefly and drain that water and add fresh ddH_2O. Incubate a couple of minutes.

3. Lift the membrane out with forceps and hang by a corner to dry. The bands will darken as the membrane dries.

Protein Concentration by Ammonium Sulfate

Calculation of grams of ammonium sulfate needed to make X% solution starting from X0% is: g=[515(X-X0)]/(100-0.27X) (for a 1:1 solution at 0^{o}C). Since most proteins will precipitate at 55% ammonium sulfate, a good value for obtaining maximum protein precipitation is 85%. For 80% precipitation, amount of solid ammonium sulfate needed is 561 g/l.

1. Place beaker of the protein solution in a cooling bath in top of a magnetic stir plate. This can be accomplished by placing the beaker within another beaker containing water or ice slurry, or by keeping the whole assembly in the refrigerator.

2. While agitating gently on a magnetic stirrer, slowly add 56.8g ammonium sulfate. Add salt more slowly as final saturation is approached, this step should take 5-10 minutes.

3. Continue stirring over night after all salt has been added.

4. Spin at 10000 x g for 10 minutes or at 3000 x g for 30 minutes.

5. Decant supernatant and resuspend precipitate in 1-2 pellet volumes of buffer. Any insoluble material remaining is probably denatured protein and should be removed by centrifugation.

6. Ammonium sulfate can be removed by dialysis, ultrafiltration, or a desalting column.
7. Stirring must be regular and gentle, stirring too rapidly will cause protein denaturation as evidenced by foaming. It is important to use a magnetic stirrer that does not generate a significant amount of heat while stirring.

8. While most proteins precipitate in the first 20 minutes after the salt is dissolved, some require several hours to completely precipitate. Precipitation should be carried out in a buffer of at least 50mM in order to compensate for a slight acidification upon dissolving ammonium sulfate.

9. The buffer should contain a chelating agent such as EDTA to remove possible traces of heavy metal cations in the ammonium sulfate which might be detrimental to the protein of interest.

10. To ensure maximal precipitation, it is best to start with a protein concentration of at least 1 mg/ml.

11. Ammonium sulfate precipitation is often a good was of stabilizing protein for storage and for concentrating protein.

12. Few proteins precipitate below 24% ammonium sulfate while most do above 80%. Ammonium sulfate precipitation results in the removal of RNA and DNA.

13. A protein's solubility may be reduced at its isoelectric point where electrostatic interactions can lead to protein aggregation and precipitation. A lower ammonium sulfate concentration will be required to precipitate a protein at its isoelectric point.

Commassie Blue Staining of an SDS-PAGE Gel

1. Place the SDS-PAGE gel into a box of an appropriate size, pour on the coomassie blue staining solution, and incubate for 30 minutes.

2. Pour off the coomassie blue staining solution, pour on the coomassie blue destaining solution, and incubate for 30 minutes.

Solutions and Buffers

- Infiltration Buffer for Vacuum Infiltration Protocol

1. Consists of 100mM Tris-HCl (pH 7.5), 10mM MgCl2, 2mM EDTA, and 0.5mM PMSF.

- Extraction Buffer for Grinding Protocol

1. Consists of 50mM Tris (pH 8.0), 2% polyvinylpyrrolidone, 0.5mM PMSF, and 5mM beta-mercaptoethanol (2ME).

- 5X Resolving Gel Buffer Stock

1. Add Tris at a concentration of 1.875M. For 1L, add 227.14g of Tris powder, or for 500 ml, add 113.57g of Tris powder.

2. pH to 8.8 with HCl.

3. Add SDS at a concentration of 0.5%. For 1L, add 5ml 10% SDS or 5g powder, or for 500ml, add 2.5ml of 10% SDS or 2.5g of powder.

- 10X Stacking Gel Buffer Stock

1. Add Tris at a concentration of 1.25M. For 100ml, add 15.14g of Tris powder.

2. pH to 6.8 with HCl.

3. Add SDS for a final concentration of 1%. For 100ml, add 5ml of 20% SDS or 1g of powder.

- 10% Ammonium Persulfate (APS)

1. Keep less than 1 month at 4^{o}C. For 100ml, add 10g of APS, of for 10ml, add 1g APS.

- 5X (10X in parentheses) Running Buffer; pH is 8.3 as a 1X solution

1. Add Tris at a concentration of 0.125M (0.25M). For 1L, add 15.15g Tris base powder (30.29g), for 10L, add 151.43g Tris base powder (302.86g).

2. Add Glycine at a concentration of 0.96M (1.9M). For 1L, add 72.1g (142.6g), or for 10L, add 720.67g (1441.34g).

3. Add SDS at a concentration of 0.5% (1.0%). For 1L, 5g powder (10g) or 50ml 10% SDS (100ml 10% SDS), or for 10L, 50g (100g) or 500ml 10% SDS (1L 10% SDS).

- 2X, 4X Sample (Loading) Buffer

1. For 10 ml final volume: add 1.25M Tris-HCl/1.0% SDS, pH 6.8 (10X stacking buffer). For, 2X buffer, add 1.0ml, and for 4X buffer add 2.0ml.

2. Add 60% glycerol, 2.0ml for 2X buffer and 4.0ml for 4X buffer.

3. Add Bromophenol Blue powder to 0.05% for both 2X and 4X buffer.

4. ddH_2O to 8.0ml, 4.0 ml for 2X buffer and 2.0ml for 4X buffer.

5. At point of use add 2-mercaptoethanol (BME). Add 4 parts 2X buffer stock plus 1 part BME (5:1) or 2 parts BME per 3 parts 4X buffer stock (5:2).

- 10X SDS-PAGE Transfer Buffer

1. 10X transfer buffer consists of 250mM Tris and 1.92M Glycine

2. In order to transfer one gel (or two gels at the same time) make 1L of 1X transfer buffer, by adding 100ml 10X transfer buffer, 200ml MeOH (for a final concentration of 20%), and 700ml ddH_2O.

- Coomassie Blue Staining Solution

1. Add the following reagents for a final concentration of Coomassie Blue R-250 (“brilliant blue” or can alternatively use Coomassie Blue G-250) to 0.025% or 25mg/100ml, MeOH to 50%, and HOAc (Glacial Acetic Acid) to 10%.

- Coomassie Blue Destaining Solution

1. Add the following reagents for a final concentration of Isopropanol to 12.5% and HOAc (Glacial Acetic Acid) to 10%.

- Commassie Blue Staining Solution

1. 2.50g Coomassie Brilliant Blue R, 455mL methanol, 455mL deionized / distilled water, and 90mL glacial acetic acid.

- Commassie Blue Destaining Solution

1. 455ml methanol, 455ml deionized/distilled water, and 90ml glacial acetic acid.

LITERATURE CITED

Arakawa, T., Chong, D.K.X., & Langridge, W.H.R. (1998) Efficacy of a food-based oral cholera toxin B subunit vaccine. *Natl. Biotechnol*. 16, 292-297.

Arntzen, C.J., Mason , H.S., Shi, J., Haq, T.A., Estes, M.K., & Clements, J.D. (1994) Production of candidate oral vaccines in edible tissues of transgenic plants. *Vaccine* 94, 339-344.

Baneyx, F. (1999) Recombinant protein expression in *Escherichia coli*. *Current Opinions in Biotechnol.* 10, 411-421.

Bardor, M., Faye, L. & Lerouge, P. (1999) Analysis of the N-glycosylation of recombinant glycoproteins produced in transgenic plants. *Trends Plant Sci.* 4, 376-380.

Bean, B., Moore, B.M., Sterner, B., Peterson, L.R., Gerding, D.N., & Balfour, H.H. Jr. (1982) Survival of influenza viruses on environmental surfaces. J. Infect Dis. 146, 47-51.

Beijerinck, M.W. (1898) Ueber ein *contagium vivum fluidum* als Ursache der Fleckenkrankheit der Tabaksblätter. *Verh. Kon. Akad. Wetensch*. 5, 3-21.

Bell, D.M. (2006) Non-pharmaceutical interventions for pandemic influenza, international measures. *Emerg. Infect. Dis*. 12, 81-87.

Bender, B.S., Rowe, C.A., Taylor, S.F., Wyatt, L.S., Moss, B., Small, J.R., & Small, P.A. (1996) Oral immunization with a replication deficient recombinant vaccinia virus protects mice against influenza. *J. Virol*. 70, 6418-6424.

Biemelt, S., Sonnewald, U., Galmbacher, P., Willmitzer, L., & Müller, M. (2003) Production of Human Papillomavirus Type 16 Virus-like Particles in Transgenic Plants. *J. Virol*. 77 (17), 9211-9220.

Black, M. & Armstrong P. (2006) An introduction to avian and pandemic influenza. *NSW Public Health Bull*. 17, 99-103.

Brennan, F.R., Bellaby, T., Helliwell, S.M., Jones, T.D., Kamstrup, S., Dalsgaard, K., Flock, J-I., & Hamilton, W.D.O. (1999) Chimeric plant virus particles administered nasally or orally induce systemic and mucosal immune responses in mice. *J. Virol*. 73,930-938.

Bridges, C.B., Kuehnert, M.J., & Hall C.B. (2003) Transmission of influenza: implications for control in health care settings. Clin. Infect. Dis. 37, 1094-1041.

Bush, R.M., Fitch, W.M. Bender, C.A., Cox, N.J. (1999) Positive selection on the H3 hemagglutinin gene of the human influenza virus. *Molecular Biology and Evolution* 16, 1457-1465.

Butler, T., Alcalde, M., Sieber, V., Meinhold, P., Schlachtbauer, C., & Arnold, F.H. (2003) Functional expression of a fungal laccase in *Saccharomyces cerevisiae* by directed evolution. *Appl. Environ. Microbiol*. 69, 987-995.

Carrillo, C., Wigdorovitz, A., Oliveros, J.C., Zamorano, P.I., Sadir, A.M., Gómez, J., Salinas, J., Escribano, J.M., & Borca, M.V. (1998) Protective immune response to foot-and-mouth disease virus with VP1 expressed in transgenic plant. J. Virol. 72, 1688-1690.

Carter III, J.E., & Langridge, W.H. (2002) Plant-based vaccines for protection against infectious diseases. *Crit. Rev. Plant*. Sci. 21, 93-109.

Casper, S.J., & Holt, C.A. (1996) Expression of the green fluorescent protein-encoding gene from a tobacco mosaic virus-based vector. *Gene* 173, 69-73.

Chanturiya, A.N., Basanez, G., Schubert, U., Henklein, P., Yewdell, J.W., & Zimmerberg, J. (2004) PB1-F2, an influenza A virus-encoded proapoptotic mitochondrial protein, creates variably sized pores in planar lipid membranes. J. Virol 78, 6304-6312.

Chikwamba, R., McMurray, J., Shou, H., Frame, B., Pegg, S-E. Scott, P., Mason, H., & Wang K. (2002) Expression of a synthetic *E. coli* heat-labile enterotoxin B sub-unit (LT-B) in maize. *Mol. Breed*. 10, 253-256.

Chin, J. (Ed.). (2000) *Control of communicable diseases manual*. Washington: American Public Health Association.

Christensen, N.D., Cladel, N.M., Reed, C.A., Budgeon, L.R., Embers, M.E., Skulsky, D.M., McClements, W.L., Ludmerer, S.W., & Jansen, K.U. (2001) Hybrid papillomavirus L1 molecules assemble into virus-like particles that reconstitute conformational epitopes and induce neutralizing antibodies to distinct HPV types. *Virology* 223, 324-334.

Christie, A.B. (1987) *Infectious Disease*. Edinburgh: Churchill Livingstone.

Clark, A.J., Bessos, H., Bishop, J.O., Brown, P., Harris, S., Lathe, R., McClenaghan, M., Prowse, C., Simons, J.P., Whitleaw, C.B. A., & Wilmut, I. (1989) Expression of human anti-haemophilic factor IX in the milk of transgenic sheep. *Bio-technology* (NY) 7, 487-492.

Clark, A.J., Bissinger, P., Bullock, D.W., Damak, S., Wallace, R., Whitelaw, C.B.A., & Yull, F. (1994) Chromosomal Position Effects and the Modulation of Transgene Expression. *Reprod. Fert. Dev*. 6, 589-598.

Cox, N.J. & Subbarao, K. (2000) Global epidemiology of influenza: past and present. Annu. Rev. Med. 51, 407-421.

Crosby, A.W. (1989) *America's forgotten pandemic. The influenza of 1918.* Cambridge: Cambridge University Press.

Daniell, H., Khan, M.S., &Allison, L. (2002) Milestones in chloroplast genetic engineering: an environmentally friendly era in biotechnology. *Trends Plant Sci.* 7, 84-91.

Daniell, H., Streatfield, S.J., & Wycoff, K. (2001) Medical molecular farming: production of antibodies, biopharmaceuticals, and edible vaccines in plants. *Trends Plant Sci*. 6, 219-226.

Dawson, W.O., Beck, D.L., Knorr, D.A., & Grantham, G.L. (1986) cDNA cloning of the complete genome of tobacco mosaic virus and production of infectious transcripts. *Proc. Natl. Acad. Sci. USA* 83, 1832-1836.

Dawson, W.O., Bubrick, P., & Grantham, G.L. (1988) Modifications of the tobacco mosaic virus coat protein gene affecting replication, movement, and symptomatology. *Phytopathology* 78, 783-789.

Dawson, W.O., & Lehto, K.M. (1990) Regulation of tobamovirus gene expression. *Adv. Virus Res*. 38, 307-342.

Deom, C.M., Lapidot, M. & Beachy, R.N. (1992) Plant virus movement proteins. Cell 69(2), 221-224.

Deom, C.M., Oliver, M.J., & Beachy, R.N. (1987) The 30-kilodalton gene product of tobacco mosaic virus potentiates virus movement. *Science* 237, 389-394.

Deom, C.M., Schubert, K.R., Wolf, S., Holt, C.A., Lucas, W.J., & Beachy, R.N. (1990) Molecular characterization and biological function of the movement protein of tobacco mosaic virus in transgenic plants. *Proc. Natl. Acad. Sci. USA* 87(9), 3284-3288.

Dowdle, W. (2006) Influenza pandemic periodicity, virus recycling, and the art of risk assessment. *Emerg. Infect. Dis*. 12 (1), 34-39.

Draper, J., Scott, R. Hamil, J. (1988) Transformation of dicotyledonous plant cells using the Ti plasmid of Agrobacterium tumefaciens and the Ri plasmid of A. rhizogenes. In: Draper J., Scott, R., Armitage, P., (eds) Plant genetic transformation and gene expression. A laboratory manual. Blackwell Scientific, Oxford, p 103.

Earn, D.J.D., Dushoff, J., & Levin S.A. (2002) Ecology and Evolution of the Flu. *Trends in Ecology and Evolution* 17, 334-340.

Emanuel, E.J., & Wertheimer, A. (2006) Who should get influenza vaccine when not all can? *Science* 312, 854-855.

Finnegan, J., & McElroy, D. (1994) Transgene inactivation: plants fight back! *Biotechnol.* 12, 833-888.

Faye, L., Fitchette-Laine, A.C., Gomord, V., Chekkati, A., Delaunay, A.M., & Driouich, A. (1993) Detection, biosynthesis, and some functions of glycans N-linked to plants secreted proteins. *Soc. Exp. Biol. Semin. Ser*. 53, 213-242.

Fitch, W.M., Bush, R.M., Bender, C.A., & Cox, N.J. (1997) Long term trends in the evolution of H(3) HA1 human influenza type A. *Proceedings of the National Academy of Sciences USA* 94, 7712-7718.

Flint, S.J., Enquist, L.W., Racaniello, V.R., & Skalka, A.M. "Principles of Virology: Molecular Biology, Pathogenesis, and Control of Animal Viruses 2nd Edition." Washington D.C.: ASM Press, 2004.

Fouchier, R.A., Munster, V., Wallenstein, A., Bestebroer T.M., Herfst, S., Smith, D., Rimmelzwaan, G.F., Olsen, B., & Osterhaus, A.D. (2005) Characterization of a novel influenza virus A hemagglutinin subtype (H16) obtained from black-headed gulls. J Virol. 79, 2814-2822.

Francis, T. Jr., Salk, J.E., & Quilligan J.J. Jr. (1947) Experience with vaccination against influenza in the spring of 1947. *Am. J. Public Health* 37, 1013-1016.

Gambaryan, A., Tuzikov, A., Pazynina, G., Bovin, N., Balish, A., & Klimov, A. (2006) Evolution of the receptor binding on influenza A (H5) viruses. *Virology* 344 (2), 432-438.

Gelvin, S.B. (2000) Agrobacterium and plant genes involved in T-DNA transfer and integration. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 51, 223-256.

Giddings, G., Allison, G., Brooks, D., & Carter, A. (2000) Transgenic plants as factors for biopharmaceuticals. *Nat. Biotechnol*. 18, 1151-1155.

Goelet, P., Lomonossoff, G.P., Butler, P.J.G., Akem, M.E., Gait, M.J., & Karn, J. (1982) Nucleotide sequence of tobacco mosaic virus RNA. *Proc. Natl. Acad. Sci. USA* 79, 5818-5822.

Gomez, N., Wigdorovitz, A., Castanon, S., Gil, F., Ordas, R., Borca, M.V., & Escribano, J.M. (2000) Oral immunogenicity of the plant-derived spike protein from swine-transmissible gastroenteritis coronavirus. *Arch. Virol*. 145, 1725-1732.

Hampson, A.W. (2002) Influenza virus antigens and antigenic drift. *Influenza*. Elsevier Science B.V., 49-86.

Hampson, A.W., and Mackenzie J.S. (2006) The influenza viruses. *Medical Journal of Australia* 185(10), S39-S43.

Hannig, G., & Makrides, S.C. (1998) Strategies for optimizing heterologous protein expression in *Escherichia coli. Trends in Biotechnol.* 16, 54-60.

Haq, T.A., Mason, H.S., Clements, J.D., & Arntzen, C. (1995) Oral immunization with a recombinant bacterial antigen produced in transgenic plants. *Science* 268, 714-716.

Harrison, B.D., & Wilson, T.M. (1999) Milestones in the research on tobacco mosaic virus. *Philos. Trans. R. Soc. B. Biol. Sci*. 354 (1383), 521-529.

Hood, E.E., & Jilka J.M. (1999) Plant-based production of xenogenic protein. *Curr. Opin. Biotechnol*. 10, 382-386.

Honys, D., Oh, S-A., Reňák, D., Donders, M., Šolcová, B., Johnson, J.A., Boudová, R., & Twell, D. (2006) Identification of microspore-active promoters that allow targeted manipulation of gene expression at early stages of microgametogenesis in *Arabidopsis*. *BMC Plant Biol*. 6: 31. Published online 2006 December 21. doi: 10.1186/1471-2229-6-31.

Harashima, S., (1994) Heterologous protein production by yeast host-vector systems. *Bioprocess Technol*. 19, 137-138.

Horsch, R.B., Fry, J.E., Hoffmann, N.L., Eichholtz, D., Rogers, S.G., & Fraley, R.T. (1985) A simple and general method for transferring genes into plants. Science 227, 1229-1231.

Huang, Z., Dry, I., Webster, D., Strugnell, R., & Wesselingh, S. (2001) Plant-derived measles virus Hemagglutinin protein induces neutralizing antibodies in mice. *Vaccine* 19 (15-16) 2163-2171.

Hunt, I. (2005) From gene to protein: a review of new and enabling technologies for multi-parallel protein expression. *Protein Expression and Purification* 40, 1-22.

Ishikawa, M., Meshi, T., Motoyoshi, F., Takamatsu, N., & Okada, Y. (1986) In vitro mutagenesis of the putative replicase genes of tobacco mosaic virus. *Nucleic Acids Res*. 14 (21), 8291-8305.

Ivanowski, D. (1892) Ueber die Mosaikkrankheit der Tabakspflanze. *St Petersb. Acad. Imp. Sci. Bull*. 35, 65-70.

Jilka, J.M., Hood, E.E., Dose, R., & Howard J.A. (1999) The benefits of proteins produced in transgenic plants. *AgBiotechNet* 1, 1-4.

Kapusta, J., Modelska, A., Figlerowicz, M., Pniewski, T., Letellier, M., Lisowa, O., Yusibov, V., Koprowski, H., Plucienniczak, A., & Legocki, A.B. (1999) A plant-derived edible vaccine against hepatitis B virus. *FASEB J*. 13, 1796-1799.

Kausche, G. A., Pfankuch, E., & Ruska, H. (1939) Die Sichtbarmachung von pflanzlichem Viren im Übermikroskop. *Naturwissenschaften* 27, 292-299.

Kearney, C.M., Chapman, S., Turpen, T.H., & Dawson, W.O. (1995) High levels of gene expression in plants using RNA viruses as transient expression vectors. *Plant Molecular Biology Manual*. Kluwer Academic, Dotdrecht.

Khandelwal, A., Lakshmi Sita, L., & Shaila, M.S. (2003) Oral immunization of cattle with Hemagglutinin protein of rinderpest virus expressed in transgenic peanut induces specific immune responses. *Vaccine* 21 (23), 3282-3289.

Kodihalli, S., Justewicz, D.M., Gubareva, L.V. & Webster, R.G. (1995) Selection of single amino acid substitution in the Hemagglutinin molecule by chicken eggs can render influenza A virus (H3): Candidate Vaccine Ineffective. *J. Virol*. 69, 4888-4897.

Korban, S.S. (2002) Targeting and expression of antigenic proteins in transgenic plants for production of edible oral vaccines. *In Vitro Cell. Dev. Biol. Plant* 38, 231-236.

Krebitz, M., Wagner, B., Ferreira, F., Peterbauer, C., Campillo, N., Witty, M., Kolarich, D., Steinkellner, H., Scheiner, O., & Breiteneer, H. (2003) Plant-based heterologous expression of Mal d 2, a thaumatin-like protein and allergen of apple (*Malus domestica*), and its characterization as an antifungal protein. *J. Mol. Biol*. 329, 721-730.

Kumagai, M.H., Donson, J., Della-Cioppa, G., & Grill, L.K. (2000) Rapid, high-level expression of glycosylated rice alpha-amylase in transfected plants by an RNA viral vector. *Gene* 245, 169-174.

Kumar, S., & Daniell, H. (2004) Engineering the chloroplast genome for hyper-expression of human therapeutic proteins and vaccine antigens. *Methods Mol. Biol*. 267, 365-383.

Kusnadi, A.R., Hood, E.E., Witcher, D.R., Howard, J.A., & Nikolov, Z.L. (1998) Production and purification of two recombinant proteins from transgenic corn. *Biotechnol. Prog*. 14, 149-155.

Kusnadi, A.R. Nikolov, Z.L., & Howard J.A (1997) Production of recombinant proteins in transgenic plants: practical applications. *Biotechnol. Bioeng*. 56, 473-484.

Lamphear, B.J., Streatfield, S.J., Jilka, J.M., Brooks, C.A., Barker, D.K., Turner, D.D., Delaney, D.E., Garcia, M., Wiggins, W., Woodard, S.L., Hood, E.E., Tizard, I.R., Lawhorn, B., & Howard, J.A. (2002) Delivery of subunit vaccines in maize seed. *J. Controlled Release* 85, 169-180.

Lauterslager, T.G.M., Florack, D.E.A., Van der Wal, T.J., Molthoff, J.W., Langeveld, J.P.M. Bosch, D., Boersma, W.J.A., & Hilgers, L.A.T. (2001) Oral immunization of naïve and primed animals with transgenic potato tubes expressing LT-B. *Vaccine* 19, 2749-2755.

Leathers, V., Tanguay, R., Kobayashi, M., & Gallie, D.R. (1993) A phylogenetically conserved sequence within viral 3' untranslated RNA pseudoknots regulates translation. *Mol. Cell Biol*. 13 (9), 5331-53347.

Lee, C.W. & Suarez D.L. (2004) Application of real-time RT-PCR for the quantitation and competitive replication study of H5 and H7 subtype avian influenza virus. *J. Virol*. Methods 119, 151-158.

Liu, H.L., Li, W.I., Lei, T., Zheng, J, Zhang, Z., Yan, X.F., Wang, Z.Z., Wang Y.L., & Si, L.S. (2005) Expression of human papillomavirus type 16 L1 protein in transgenic tobacco plants. *Acta. Biochim. Biophys. Sin (Shanghai)* 37 (3), 153-158.

Makrides, S.C. (1996) Strategies for achieving high-level expression of genes in *Escherichia coli*. *Microbiol*. 60, 512-538.

Mandell, G., Douglas, J., & Bennet, R. (Eds.) (2005) *Principles and practice of infectious diseases*. Sixth edition. Philadelphia: Churchill Livingstone.

Marquet-Blouin, E., Bouche, F.B., Steinmetz, A., & Muller, C.P. (2003) Neutralizing immunogenicity of transgenic carrot (*Daucus carota* L.)-derived measles virus hemagglutinin. *Plant. Mol. Biol*. 51, 459-469..

Mason, H.S., & Arntzen, C.J. (1995) Transgenic plants as vaccine production systems. *Trends Biotechnol*. 13, 388-392.

Mason, H.S., Ball, J.M., Shi, J.J., Jiang, X., Estes, M.K., & Arntzen, C.J. (1996) Expression of Norwalk virus capsid protein in transgenic tobacco and potato and its oral immunogenicity in mice. *Proc. Natl. Acad. Sci*. USA 93, 5335-5340.

Mason, H.S., Haq, T.A., Clements, J.D., & Arntzen, C.J. (1998) Edible vaccine protects mice against *Escherichia coli* heat-labile enterotoxin (LT): potatoes expressing a synthetic LT-B gene. *Vaccine* 16, 1336-1343

Mason, H.S., Lam, D.M., Arntzen, C.J. (1992) Expression of hepatitis B surface antigen in transgenic plants. *Proc. Natl. Acad. Sci. USA* 89, 11745-11749.

Matrosovich, M.N., Gambaryan, A.S., Teneberg, S., Piskarev, V.E., Yamnikova, S.S., Lvov, D.K., Robertson, J.S., & Karlsson, K.A. (1997) Avian influenza A viruses differ from human viruses by recognition of sialyloligosaccharides and gangliosides and by a higher conservation of the HA receptor-binging site. *Virology* 233, 224-234.

Mayer, A. (1886) Ueber die Mosaikkrankheit des Tabaks. *Landw. Vers. Sta.* 32, 451-467.

Modelska, A., Dietzschold, B., Sleysh, Z.F., Fu, Z.F., Steplewski, K., Hooper, D.C., Koprowski, H., & Yusibov, V. (1998) Immunization against rabies with plant-derived antigen. *Proc. Natl. Acad. Sci*. USA 95, 2481-2485.

Mor, T.S., Gomez-Lim, M.A., & Palmer, K.E. (1998) Perspective: edible vaccines-a concept coming of age. *Trends Microbiol*. 6, 449-453.

Musiychuk, K., Stephenson, N., Hong, B., Farrance, C.E., Orozovic, G., Bordelius, M., Brodelius, P., Horsey, A., Ugulava, N., Shamloul, A-M., Mett, V., Rabindran, S., Streatfield, S.J., & Yusibov, V. (2007) A launch vector for the production of vaccine antigens in plants. *Influenza and Other Respiratory Viruses* 1, 19-25.

Ni, M., Cui, D., Einstein, J., Narasimhulu, S., Vergara, C.E., & Gelvin, S.B. (1995) Strength and tissue specificity of chimeric promoters derived from the octopine and mannopine synthase genes. *The Plant Journal*. 7(4), 661-676.

Palomares, LA., Estrada-Mondaca, S., & Ramirez OT. (2004) Production of recombinant proteins: challenges and solutions. *Methods Mol. Biol.* 267, 15-52.

Payungporn, S., Phakdeewirot, P., Chutinimitkul, S., Theamboonlers, A., Keawcharoen, J., Oraveerakul, K., Amonsin, A., & Poovorawan, Y. (2004) Single-step multiplex reverse transcription-polymerase chain reaction (RT-PCR) for influenza A virus subtype H5N1 detection. *Viral Immunol.* 17(4), 588-593.

Pogue, G.P., Lindbo, J.A., Dawson, W.O., & Turpen, T.H. (1998) "Plant Molecular Biology Manual" (Gelvin, S.B., & Schilperoort, R.A., Ed.) Kluwer Academic Publishers, Dordrecht, Netherlands.

Potter, C.W. (1998) Chronicle of influenza pandemics. *Textbook of Influenza.* ed. Nicholson, K.G., Webster, R.F., and Hay, A.J. Oxford: Blackwell Science Ltd. 3-18.

Potter, C.W. (2001) A history of influenza. *J. of Applied Microbio.* 91, 572-579.

Potter, C.W., & Oxford, J.S. (1979) Determinants of immunity to influenza virus infection in man. *British Med. Bull*. 35, 69-75.

Powers, D.C., Smith, G.E., Anderson, E.L., Kennedy, D.J., Hackett, C.S., Wilkinson, B.E., Volvovitz. F., Belshe, R.B. & Treanor, J.J. (1995) Influenza A virus vaccines containing purified recombinant H3 Hemagglutinin are well tolerated and induce protective immune responses in healthy adults. *J. Infect. Dis*. 171, 1595-1599.

Richter, L.J., Thanavala Y., Arntzen, C.J., & Mason, H.S. (2000) Production of hepatitis B surface antigen in transgenic plants for oral immunization. *Nat. Biotechnol*. 18, 1167-1171.

Roodveldt, C., Aharoni, A., & Tawfik, D.S. (2005) Directed evolution of proteins for heterologous expression and stability. *Current Opinion in Structural Biology* 15, 50-56.

Saelens, X., Vanlandschoot, P., Martinet, W., Maras, M., Neirynck, S., Contreras, R., Fiers, W., & Min Jou, W. (1999) Protection of mice against a lethal influenza virus challenge after immunization with yeast-derived secreted influenza virus Hemagglutinin. *Eur. J. Biochem* 260, 166-175.

Santa Cruz, S., Chapman, S., Roberts, A.G., Roberts, I.M., Prior, D.A.M., & Oparka, K.J. (1996) Assembly and movement of a plant virus carrying a green fluorescent protein overcoat. *Plant Biology* 93, 6286-6290.

Satyavathi, V.V., Prasad, V., Abha khandelwal, Shaila, M.S., & Lakshmi Sita, G. (2003) Expression of Hemagglutinin protein of Rinderpest virus in transgenic pigeon pea [*Cajanus cajan* (L.) Millsp.] plants. *Plant Cell Rep* 21, 651-658.

Scholthof, H.B., Scholthof, K.B., & Jackson, O.A. (1996) Plant virus gene vectors for transient expression of foreign proteins in plants. *Annu. Rev. Phytopathol*. 34, 299-323.

Shaw, J.G. (1996) Plant Viruses. Third ed. In "Fundamental Virology" eds. Fields, B.N., Knipe, D.M., & Howley, P.M. Lippincott-Raven, New York. P. 367-401.

Simeckova-Rosenberg, J., Yun, Z., Wyde, P.R. & Atassi, M.Z. (1995) Protection of mice against lethal viral infection by synthetic peptides corresponding to B-and T-cell recognition sites of influenza A Hemagglutinin. *Vaccine* 13, 927-932.

Slepushkin, V.A., Katz, J.M., Black, R.A., Gamble, W.C., Rota, P.A., & Cox, N.J. (1995) Protection of mice against influenza A virus challenge by vaccination with baculovirus-expressed M2 protein. *Vaccine* 13, 1399-1402.

Smith, D.J., Lapedes, A.S., de Jong, J.C., Bestebroer, T.M., Rimmelzwaan, G.F., Osterhaus, A.D.M.E., Fouchier, R.A.M. (2004) Mapping the antigenic and genetic evolution of influenza virus. *Science* 305, 371-376.

Spackman, E., Senne, D.A., Myers, T.J., Bulaga, L.L., Garber, L.P., Perdue, M.L., Lohman, K., Daum, L.T., & Suarez, D.L. (2002) Development of a real-time reverse transcription PCR assay for type A influenza virus and the avian H5 and H7 hemagglutinin subtypes. *J. Clin. Microbiol.* 40(9), 3256-3260.

Stanley, W.M. (1935) Isolation of a crystalline protein possessing the properties of tobacco-mosaic virus. *Science* 81 (2113), 644.

Starr, I. (1976) Influenza in 1918: recollections of the epidemic in Philadelphia. *Annals of Internal Medicine* 85, 516-518.

Stoger, E., Sack, M., Fischer, R., & Christou, P. (2002) Plantibodies: applications, advantages, and bottlenecks. *Curr. Opin. Biotechnol*. 13, 161-166.

Stohr, K. (2003) Overview of the WHO Global Influenza Programme. Dev. Biol (Basel) 115, 3-8.

Streatfield, S.J., & Howard, J.A. (2003) Plant-based vaccines. *Int. J. Parasitol.* 33 (5-6), 479-493.

Streatfield, S.J., Lane, J.R., Brooks, C.A., Barker, D.K., Poage, M.L., Mayor, J.M., Lamphear, B.J., Drees, C.F., Jilka, J.M., Hood, E.E., & Howard, J.A. (2003) Corn as a production system for human and animal vaccines. *Vaccine* 21, 812-815.

Tacket, C.O., Mason, H.S., Losonsky, G., Clements, J.D., Levine, M.M., Arntzen, C.J. (1998) Immunogenicity in humans of a recombinant bacterial antigen delivered in a transgenic potato. *Nat. Med*. 4, 607-609.

Tacket, C.O., Mason, H.S., Losonsky, G., Estes, M.K., Levine, M.M., Arntzen, C.J. (2000) Human immune responses to a novel Norwalk virus vaccine delivered in transgenic potatoes. *J. Infect. Dis*. 182, 302-305.

Tacket, C.O., Pasetti, M.F., Edelman, R., Howard J.A., & Streatfield, S. (2004) Immunogenicity of recombinant LT-B delivered orally to humans in transgenic corn. *Vaccine* 22, 4385-4389.

Taubenberger, J., & Morens, D. (2006) 1918 Influenza: the mother of all the pandemics. *Emerg. Infect. Dis.* 12 (1), 15-22.

Taubenberger, J., Reid, A., Lourens, R., Wang, R., Jin, G., & Fanning T. (2005) Characterization of the 1918 influenza virus polymerases genes. Nature 437, 889-893.

Thanavala, Y., Mahoney, M., Pal, S., Scott, A., Richter, L., Natarajan, N., Goodwin, P., Arntzen, C.J., & Mason, H.S. (2005) Immunogenicity in humans of an edible vaccine for Hepatitis B. *Proc. Natl. Acad. Sci. USA* 102, 3378-3382.

Thanavala, Y., Yang, Y-F., Lyons, P., Mason, H.S., & Arntzen, C. (1995) Immunogenicity of transgenic plant-derived hepatitis B surface antigen. *Proc. Natl. Acad. Sci USA* 92, 3358-3361.

Tumpey, T.M., Basler C.F., Aguilar, P.V., Zeng, H., Solorzano, A., Swayne D.E., Cox, N.J., Katz, J.M., Taubenberger, J.K., Palese, P., & Garcia-Sastre, A. (2005) Characterization of the reconstructed 1918 Spanish influenza pandemic virus. Science 310 (5745), 77-80.

Turpen, T.H., Turpen, A.M., Weinzettl, N., Kumagai, M.H., & Dawson,W.O. (1993) Transfection of whole plants from wounds inoculated with Agrobacterium tumefaciens containing cDNA of tobacco mosaic virus. *J. Virol. Methods* 42, 227-239.

Twyman, R.M., Schilliberg, S., & Fischer, R. (2005) Transgenic plants in the biopharmaceutical market. *Expert Opin. Emerg*. Drugs 10, 185-218.

Ulmer, J.B., Donnelly, J.J., Parker, S.E., Rhodes, G.H., Felgner, P.L., Dwarki, V.J., Gromkowski, S.H., Deck, R.R., De Witt, C.M., Friedman, A., Hawe, L.A., Leander, K.R., Martinez, D., Perry, H.C., Shiver, J.W., Montgomery, D.L. & Liu, M.A. (1993) Heterologous protection against influenza by injection of DNA encoding a viral protein. *Science* 259, 1745-1749.

Varsani, A., Williamson, A-L., Rose, R.C., Jaffer, M., & Rybicki, E.P. (2003) Expression of *Human papillomavirus* type 16 major capsid protein in transgenic *Nicotiana tabacum* cv. Xanthi. *Arch. Virol*. 148, 1771-1776.

Verhoeyen, M., Fang, R., Min Jou, W., Devos, R., Huylebroeck, D., Saman, E. & Fiers, W. (1980) Antigenic drift between the Hemagglutinin of the Hong Kong influenza strains A/Aichi/2/68 and A/Victoria/3/75. *Nature* 286, 771-776.

Webster, D.E., Thomas, M.C., Huang, Z., & Wesselingh S.L. (2005) The development of a plant-based vaccine for measles. *Vaccine* 23 (15), 1859-1865.

Webster, R.G., Bean, W.J., Gorman, O.T., Chambers, T.M. & Kawaoka, Y. (1992) Evolution and ecology of influenza A viruses. Microbiol Rev. 56, 152-179.

Webster R.G., & Laver, W.G. (1971) Antigenic variation in influenza virus. Biology and chemistry. *Prog. Med. Virol.* 13, 271-338

Webster, R.G., Laver, W.G., Air, G.M. & Schild, G.C. (1982) Molecular mechanisms of variation in influenza viruses. *Nature* 296, 115-121.

Weiler, K.S., & Wakimoto, B.T. (1995) Heterochromatin and gene expression in *Drosophila*. *Annu. Rev. Genet*. 29, 577-605.

Whitelam, G.C. (1995) The production of recombinant proteins in plants. *J. Sci. Food Agric*. 68, 1-9.

Whitelaw, C.B.A., Archibald, A.L., Harris, S., McClenaghan, M., Simons, J.P., & Clark, A.J. (1991) Targeting expression to the mammary gland: intronic sequences can enhance the efficiency of gene expression in transgenic mice. *Transgenic Res.* 1, 3-13.

Wigdorovitz, A., Carrillo, C., Santos, M.J.D., Trono, K., Peralta, A., Gómez, M.C., Rios, R.D., Franzone, P.M., Sadir, A.M., Escribano, J.M., & Borca, M.V. (1999a) Induction of a protective antibody response to foot and mouth disease virus in mice following oral or parenteral immunization with alfalfa transgenic plants expressingthe viral structural protein VP1. *Virology* 255, 347-353.

Wigdorovitz, A., Filgueira, D.M.P., Robertson, N., Carrillo, C., Sadir, A.M., Morris, T.J., & Borca, M.V. (1999b) Protection of mice against challenge with foot and mouth disease virus (FMDV) by immunization with foliar extracts from plants infected with recombinant tobacco mosaic virus expressing the FMDV structural protein VP1. Virology 264, 85-91.

Wong, S.S.Y., and Yuen, K-Y. (2006) Avian Influenza Virus Infections in Humans. CHEST 129, 156-168.

World Health Organization (WHO). (2005) Avian influenza: assessing the pandemic threat. The World Health Organization. Available at: www.who.int/csr/disease/influenza/WHO_CDS_2005_29/en/index.html. Accessed on May, 10, 2007.

Yusibov, V., Hooper, D.C., Spitsin, S.V., Fleysh, N., Kean, R.B., Mikheeva, T., Deka, D., Karasev, A., Cox, S., Randall, J., & Koprowski, H. (2002) Expression in plants and immunogenicity of plant virus-based experimental rabies vaccine. *Vaccine* 20, 3155-3164.

Yusibov, V., Rabindran, S., Commandeur, U., Twyman, R.M., &Fischer, R., The potential of plant virus vectors for vaccine production. *Drugs R. D*. 7, 203-217.

Yusibov, V., Shivprasad, S., Turpen, T.H., Dawson, W., & Koprowski, H. (1999) Plant viral vectors based on tobamoviruses. *Curr. Top. Microbiol. Immunol.* 240, 81-96.

Zeenko, V.V., Ryabova, L.A., Spirin, A.S., Rothnie, H.M., Hess, D., Browning, K.S., & Hohn, T. (2002) Eukaryotic elongation factor 1A interacts with the upstream pseudoknot domain in the 3' untranslated region of tobacco mosaic virus RNA. *J. Virol.* 76 (11), 5678-5691.