# Chapter I
## Literature Review

With advances in agricultural biotechnology and plant molecular biology, a considerable effort has been focused on genetic engineering for enhancement of plant quality, protection and production of new products. During the last decade, using transgenic plants to express recombinant proteins for pharmaceutical use has become very attractive. Plants have the potential to provide a large amount of inexpensive and safe proteins for disease diagnosis and therapy. Transgenic plants have been used to produce cytokines, hormones, monoclonal antibodies, industrial and therapeutic enzymes, and vaccines (reviewed in Miele, 1997; Cramer *et al.*, 1999). In the near future, it may be possible that recombinant vaccine antigens will be made in plants and delivered as oral doses via food or food supplements.

Traditional vaccines include killed or attenuated organisms or strains of a pathogen whose host differs from the vaccinated species (Richter and Kipp, 1999). With the help of rapidly developing genetic engineering techniques, recombinant subunit vaccines have become available as alternatives to these traditional approaches. A subunit vaccine is composed of one or more proteins or glycoproteins from the pathogen that induces a protective immune response in the host. Therefore, subunit vaccines do not have the capacity to induce disease but rather prevent disease. Conventional technologies have a certain amount of risk associated with contamination e.g., live, pathogenic animal viruses and bacteria. Plants on the other hand do not serve as direct hosts for human or animal pathogens (Moffat, 1995).

Currently, commercial systems for recombinant protein production include yeast or bacterial hosts or mammalian cell culture systems. Bacteria lack the ability for extensive post-translational modifications that are required by most eukaryotic proteins for complete antigenicity (Fischer *et al.*, 1999). In order to produce subunit vaccines, recombinant proteins expressed by bacteria, yeast and mammalian cells need to be extensively purified to remove host proteins and compounds and thus increase the cost of recombinant vaccines (Moffat, 1995). The costs of currently available commercial vaccines are prohibitively expensive for most people in developing counties where the vaccines are needed most. The basic costs of most vaccines arises from not only purification, but also packing, delivery, refrigeration during shipping and storage, the use of needles and syringes as well as the need for trained people to administer injections (Moffat, 1995). These costly procedures keep effective vaccines out of the reach of millions of people and leave them susceptible to preventable diseases (Hausdorff, 1996). These economic considerations also make it less likely for the worldwide vaccination of livestock and poultry to protect against preventable infections (Richter and Kipp, 1999). Transgenic plants engineered to express and present protective antigens and delivered orally through food or feed without purification or with minimal processing provides an exciting opportunity to meet these health needs cost-effectively.

Given these considerations, transgenic plants as edible vaccines represent a promising option for human, livestock and poultry population world - wide.

**Strategies to make transgenic plants**

The ability to effectively generate transgenic plants has revolutionized plant biology. A foreign gene can be integrated into a plant genome and its expression will lead to the production a novel protein and/or activity in plants. Generation of transgenic plants involves two processes, introduction/integration of new DNA (transformation) and production of a plant from the newly transformed cell (regeneration). A variety of plant transformation methods have been developed (Owen and Pen, 1996). The most widely used methods are *Agrobacterium*-mediated gene transfer and transformation via particle bombardment. *Agrobacterium tumefaciens* is a soil bacterium that has evolved a natural mechanism of transferring a DNA segment called T-DNA from its own plasmid DNA to a plant cell and integrating the T-DNA into the plant genome (Sheng and Citovsky, 1996). By inserting foreign "genes of interest" and an antibiotic selectable marker within the T-DNA, *Agrobacterium* can be used to efficiently integrate new genes into plants. Transformation simply involves co-cultivation of small leaf sections with the recombinant *Agrobacterium* and then regeneration of "plantlets" on selective media that kills the *Agrobacterium* and non-transformed plant cells but encourages formation of shoots and then roots (Fig. 1). The resulting transgenic plants generally contain one to four copies of the new DNA randomly inserted into the genome and these genes now segregate in a normal Mendelian fashion. Particle bombardment was initially developed for transformation of plant species that do not naturally interact with *Agrobacterium* and involves coating the foreign DNA onto gold or tungsten microspheres and accelerating the beads into plant cells or tissues using a device termed a "gene gun". The DNA can be transiently expressed in the cells or can be stably integrated into the chromosomes yielding transgenic plants (Hammond, 1999). This method is successful for a range of species including tobacco (Klein *et al*., 1988), soybean (McCable *et al*., 1988), maize (Fromm *et al*., 1990; Gordon - Kamm *et al*., 1990), rice (Chtiston *et al*., 1991), barley (Wan et al., 1994) and wheat (Vasil *et al*., 1992; Weeks *et al*., 1993).

Virus-mediated transgene expression has also emerged as an alternative and more rapid means of generating recombinant proteins, including vaccines, in plants. This strategy does not involve production of a stable transgenic plant, but entails modifying the genome of a plant virus and then infecting a susceptible host plant. The virus spreads throughout the plant and directs the expression of the foreign protein in the infected cells. Tobacco mosaic virus and cowpea mosaic virus have been most widely used for these applications (Ma *et al.*, 1999; Lomonossoff and Hamilton, 1999). In general, the entire viral genome is engineered into a bacterial transcription vector with a cloning site designed within the coding region of the viral coat or capsid protein for insertion of foreign DNA. RNA transcription is then used to generate infective viral RNAs. Upon infection into a susceptible host, intact viral particles are produced that contain the foreign epitope displayed on the surface of the viral capsid (Della-Cioppa *et al.*, 1996). These "chimeric viruses" can be easily purified by homogenization and centrifugation, and used as a vaccine, or for large-scale inoculation of plants to yield more viruses. The advantages of the virus-based systems are the high levels of foreign protein production that are achieved and the rapid "engineering" and testing procedure (Kumagai *et al.*, 1993). Because plant viruses cannot replicate in animals (and, in fact, have been part of human and livestock diets since we began eating plants), there is no apparent need to

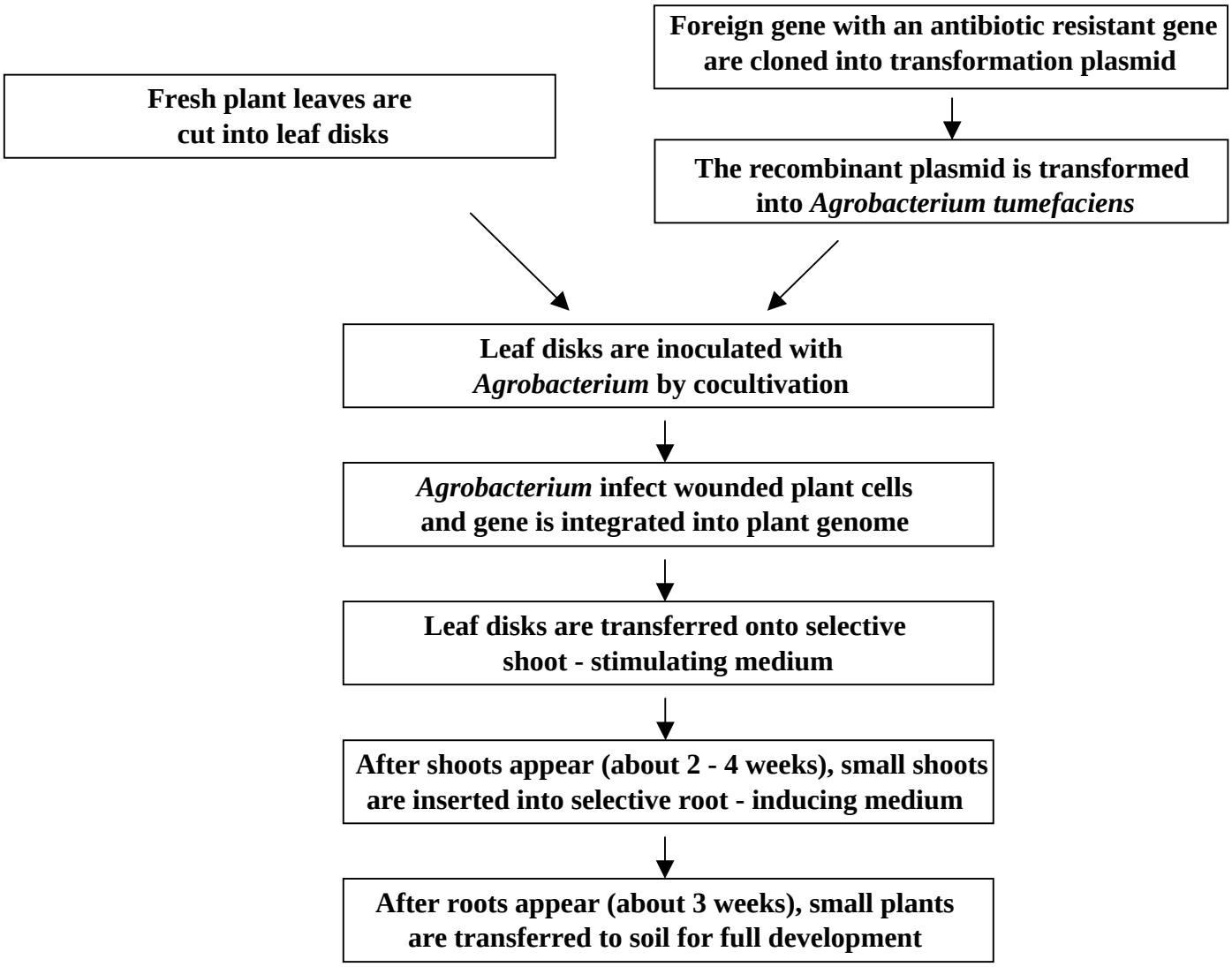



Fig. 1. generation of transgenic plant using *Agrobacterium* – mediated transformetion

worry about potential infectious materials. In addition, viral particles serve as excellent immunogens. Two factors may limit the widespread use of viral-mediated vaccine production: 1) the small amount of foreign DNA that can be inserted into the some viral genomes (Sugiyama *et al.*, 1995) and 2) issues of environmental release of active recombinant viruses. Transgenic plants have the advantage of stable insertion of the foreign gene into plant nuclear chromosome. The breeding line is stable from generation to generation. The edible plant part can be delivered directly as food or feed without purification and, because large amounts of DNA can be introduced, there is the opportunity to produce very large antigens and multi-component vaccines.

**Transgenic plants as oral vaccines**

In 1990, the Children's Vaccine Initiative (Mitchell *et al.*, 1993) called for novel vaccines to be made more widely available around the world. They encouraged the development of low cost oral vaccines as well as multicomponent vaccines that can deliver protections against several infections at once. Oral vaccines are easy to administer and they may directly stimulate the specific humoral and cellular response on the mucosal surface that is the first barrier against various infectious pathogens (Phalipon and Sansonetti, 1999).

Transgenic plants may provide a feasible system to produce low cost oral vaccines. Plants are capable of synthesizing and assembling very complex proteins into a form that provides the required bioactive or immunogenic response (Cramer *et al*., 1999). Furthermore, using transgenic plants to produce vaccines eliminates the potential for animal virus contamination from animal sources (Moffat, 1995). Plants do not serve as hosts for human pathogens and so assures the safety of vaccination. Plants have the potential for scale-up production with much lower costs. It is not necessary to use special media, toxic chemicals or expensive equipment to grow plants. Vaccine-expressing breeding lines can be distributed by seeds and harvested through conventional techniques. In addition, transgenic plants have the potential to deliver multiple antigen subunits to protect against several diseases at one time (Wong *et al.*, 1997).

**Edible vaccines for humans**

Up to now, tobacco and potatoes are the most commonly used transgenic models because they are relatively easy to genetically engineer. A variety of antigens from pathogens causing human disease have been expressed in transgenic plants (see Table 1) and their immunogenicity has been tested in mice or, in some cases, in human clinical trials. The first reported antigen produced in transgenic plants was the cell surface adhesion protein antigen A (SpaA) from *Streptococcus mutans*, a major bacterial pathogen causing tooth decay. SpaA, an 185 kDa protein, was expressed at a level of up to 0.02% of total leaf protein, demonstrating the potential of using transgenic plant to produce vaccine antigens (Curtiss and Cardineau, 1989).

The heat labile enterotoxin (LT) is one of the toxins secreted by enterotoxic *Escherichia coli* and causes diarrhea. LT is composed of a toxic A subunit and a pentameric B (LT-B) subunit responsible for binding to the GM1 gangliosides on small intestine epithelial cells. Cholera toxin (CT) from *Vibrio cholerae* also causes diarrhea

**Table 1. Candidate vaccines for human diseases**

| Target | Strategies to make plant-based vaccine | Reference |
|---|---|---|
| Human HIV | tobacco mosaic virus | Sugiyama *et al.,* 1995 |
| Malaria | tobacco mosaic virus | Turpen *et al.,* 1995 |
| Human influenza | cowpea mosaic virus | Sugiyama *et al.,* 1995 |
| Measles | cowpea mosaic virus | Lomonossoff *et al.,* 1999 |
| Cell surface-adhesion protein for tooth decay | transgenic tobacco | Curtiss *et al.,* 1990 |
| Hepatitis B | transgenic tobacco | Mason *et al.,* 1992 |
| Norwalk viral enteritis | transgenic tobacco and potato | Mason *et al.,* 1996 |
| Cholera toxin | transgenic potato | Arakawa *et al.,* 1997 |
| LT-B | transgenic tobacco | Haq *et al.,* 1995 |

and is structurally and immunogenically highly homologous to LT. Immunization with CT-B or LT-B can give protection against both diseases (Haq *et al.*, 1995); both toxins also function as oral adjuvants. Recombinant LT-B was expressed in tobacco and potato (Haq *et al.*, 1995). The plant-synthesized LT-B formed pentameric structures, bound to GM1 gangliosides, and was recognized by anti-LT serum. Mice fed raw transgenic potato tubers developed both serum IgGs and mucosal IgAs capable of neutralizing *E. coli* LT toxin *in vitro*. To increase LT-B accumulation in tubers, the LT-B gene was further modified by optimizing plant-preferred codons, targeting, and expression using a tuber specific promoter to yield 10-20 μg recombinant LT-B per gram fresh tuber (Mason *et al.*, 1998). Human volunteers ingesting raw potato tuber slices without adjuvant showed four - fold increases in anti-LT serum IgG (10 of 11 volunteers) and anti - LT mucosal IgA (5 of 10 volunteers) (Tacket *et al.*, 1998). These results indicated both systemic and mucosal immune responses and demonstrated a fundamental "proof of concept" for transgenic plant-derived oral vaccines.

Hepatitis B surface antigen (HbsAg) was the first commercial recombinant vaccine derived from yeast and is normally delivered by injection. Because HbsAg self-assembled into virus-like particles, it is a good antigen to test its oral immunogenicity. The HbsAg was expressed in tobacco and potato in 1992 (Mason *et al.*, 1992) and shown to assemble into virus-like particles similar to those produced by yeast. After a crude HbsAg-containing extract was administrated into mice, the mice developed specific humoral immune responses to the plant-synthesized HbsAg (Thanavala *et al.*, 1995). These immune responses were comparable to mice immunized with yeast-derived HbsAg. This was the first report of using transgenic plants to produce a whole virus-like particle and to elicit a successful immune response. Human trials have been initiated for both antigens (C. Artzen, Cornell University, personal communication).

Norwalk virus causes epidemic acute gastroenteritis in animals and humans (Kaplan *et al.*, 1982). The virus capsid protein, which is produced in recombinant baculovirus-infected inset cells, self-reassmembled into virus-like particles (i-rNV) (Ball *et al.*, 1996). When produced in tobacco and potato, the capsid protein also assembled into virus-like particles (t-rNV) that appear identical under electron microscopy as i-rNV (Mason *et al.*, 1996). Following immunoblot analysis, t-rNV and i-rNV comigrate at same molecular mass. When extracts of tobacco plant leaves were given mice by gavage, both serum and mucosal Ab responses were detected. However, of 20 mice fed potato tubers that express capsid protein, only 11 developed serum IgG response and only 1 had a specific mucosal IgA response. It was suggested that the potato tuber produced less virus-like particles which is more stable in the stomach and should be more immunogenic (Jiang, 1992). It may be necessary to use a larger oral dose to stimulate effective IgA response.

Strategies involving genetically modified plant viruses to produce vaccine antigens in plant has also achieved successes. Tobacco mosaic virus (TMV) is a well-characterized RNA virus. After infection, the virus can accumulate up to 50% by mass of dry plant material (Ma *et al.*, 1999). Epitopes from malaria have been expressed on the surface of the TMV capsid with high yields of 0.4-1.2 mg/g fresh plant weight (Turpen *et al.*, 1995).

Immunogenicity tests in mice demonstrated that the malaria epitopes were effectively presented on the surface of the viral particle. Epitopes from *Haemophilus. influenzae* and human immunodeficiency virus (HIV) have also been expressed at high levels on the surface of TMV and/or CPMV (Lomonossoff and Hamilton, 1999).

**Edible vaccines for livestock including poultry**

The development of low-cost feed-based vaccines has significant implications for livestock and poultry and could eventually impact disease management in wildlife and conservation programs as well. As shown in Table 2, a variety of target antigens for animal diseases are also under research and development. Transmissable gastroenteritis virus (TGEV) of swine causes diarrhea in newborn piglets (Gomez *et al.,* 1998). Oral vaccination with adenovirus and *Salmonella*-vectored TGEV glycoprotein S induced both systemic and mucosal antibody immunity and conferred protection against the disease in piglets (Smerdou *et al.,* 1996; Torres *et al.,* 1996). The full-length glycoprotein S was expressed in the model plant, *Arabidopsis thaliana*, and leaf extract containing the glycoprotein S was injected into mice. Specific serologic responses were detected and the antibody effectively neutralized the virus *in vitro* (Gomez *et al.,* 1998). This confirmed the feasibility of using plant-produced glycoproteins as a source of recombinant antigen for vaccine production but further tests are required to establish the oral efficacy.

The structural protein VP1 of foot-and mouth disease virus has been expressed in transgenic *Arabidopsis* (Carrillo *et al.,* 1997) and alfalfa (Wigdorovitz *et al.,* 1998). Mice immunized with plant-synthesized VP1 protein developed specific antibody responses and were protected against experimental virulent challenge.

The cowpea mosaic virus (CPMV) system has been used to produce plant viruses displaying several animal disease epitopes (Table 2). Dalsgaard *et al.* (1997) expressed an epitope from the mink enteritis virus capsid protein on the CPMV capsid surface. The yield of the chimeric virus particles was about 1-1.2 mg/g of fresh plant material. After the administration of the purified virus mixed with adjuvant, mink developed specific antibody response to the epitope and 11 of 12 immunized mink were fully protected against the disease.

**Hemorrhagic enteritis of turkeys**

Hemorrhagic enteritis (HE) is an acute disease, characterized by depression, bloody diarrhea and sudden death (Pierson and Domermuth, 1997). HE occurs in commercial turkey flocks 4 – 13 weeks of age and older (Domermuth and Gross, 1984). The mortality is usually observed 7 – 10 days after infection (Gross and Moore, 1967). Younger birds are usually resistant to the disease as a result of the presence of maternal antibodies (Domermuth and Gross, 1984). HE is widespread and has been observed throughout the world where turkeys are raised. This disease is transmitted by oral contact with infected material; likely introduced by persons or vermin carrying infected litter. There were losses of $3 million dollars per year in USA before the development of vaccines (Van den Hurk *et al.,* 1994).

**Table 2. Candidate vaccines for animal diseases**

| Target | Strategies to make plant-based vaccine | Reference |
|---|---|---|
| Canine parvoviral enteritis | cowpea mosaic virus | Lomonossoff *et al.,* 1999 |
| Mink enteritis | cowpea mosaic virus | Dalsgaard *et al.,* 1997 |
| Rabies | tobacco mosaic virus | Modelska *et al.,* 1998 |
| Bovine viral diarrhea | transgenic barley | Gilbert *et al.,* 1999 |
| Canine papilloma | transgenic tobacco | Allina *et al.,* 1999 |
| Foot-and- mouth disease | transgenic arabidoposis<br>transgenic alfalfa | Carrillo *et al.,* 1998<br>Wigdorovitz *et al.,* 1999 |
| Hemorrhagic enteritis of turkeys | transgenic tobacco | Tian *et al.,* unpublished |
| Hemorrhagic enteritis of Rabbits | transgenic potato | Castanon *et al.,* 1999 |
| Porcine reproductive and respiratory syndrome | transgenic tobacco | Rymerson *et al.,* 1999 |
| Transmissible gastroenteris of swine | transgenic arabidoposis | Gomez *et al.,* 1998 |

HE is caused by HEV, which is classified as a member of avian adenovirus group (type) II within the family *Adenoviridea* and the genus *Aviadenovirus* (Pierson and Domermuth, 1997). The virus is a nonenveloped, DNA virus with icosahedral symmetry. The 96 kDa hexon, 51/52 kDa penton base, and 29 kDa penton fiber proteins (van den Hurk, 1992) are characteristic and major proteins comprising the virus outer capsid. Pentons, including one penton base and a single fiber, are located at the 12 vertices of the icosahedron and each penton base is surrounded by five hexons (van den Hurk, 1992).

Fiber is a structural protein with the crucial function of recognizing and attaching to host receptors in the initial stage of infection. Fiber is composed of a tail, a shaft and a globular head (knob) (van den Hurk, 1992). The N-terminus of the polypeptide chain in the fiber is located in the tail region (Devaux *et al*., 1987) and associates with the penton base in a noncovalent manner. The C-terminus is in the knob region that interacts with viral receptors (Devaux *et al*., 1990). Two forms of vaccines are now widely used to prevent HE. One is splenic vaccine produced *in vivo* by orally infecting turkeys with avirulent HE virus (HEV-A, Domermuth and Larson, 1984); the spleens are collected 3-5 days post exposure and the crude homogenate containing live HEV-A is administered in drinking water (Domermuth *et al.*, 1977; Thorsen *et al.*, 1982). The other vaccine is the HEV-A propagated *in vitro* in RP19 cell culture (Nazerian and Fadly, 1987). Both of these vaccines induce protection against HE in turkeys (Fadly *et al.*, 1985). However, immunizations with these live virus vaccines have been shown to be immunosuppressive and predispose turkeys to secondary bacterial infections, notably *E. coli* (Pierson and Domermuth, 1997).

Currently, the strategy of using transgenic plants to develop a protective vaccine against HE and avoid the side – effects with current HE vaccines is being explored. Research showed that hexon purified from virus particles protected turkeys against HE challenge. Two immunizations of only 0.3 μg hexon / dose can completely protect turkeys from the disease (van den Hurk *et al.*, 1993). Antibodies against fiber and penton base can also neutralize the virus and offer protection (van den Hurk *et al.*, 1993). The hypothesis tested by this thesis research is to determine if a plant could be used to synthesize the HEV fiber protein and produce sufficient antigen to stimulate protective HE antibodies when administrated to turkeys.

Based on the deduce amino acid sequence of the HEV fiber gene (van den Hurk, 1992), the HEV fiber has a predicted protein molecular mass of 49 kDa and has six potative N-linked glycosylation sites (N-X-S/T). The molecular size of fiber purified from HEV viral particles is 29 kDa suggesting posttranslational processing of the gene product.

To introduce the fiber gene into plants, the coding region of the HEV fiber gene was fused to either a constitutive plant promoter (35S) or a wound inducible promoter (MeGA$^{TM}$) in plant transformation vectors. The constructs were separately introduced into tobacco using *Agrobacterium*-mediated transformation. Approximately sixty transgenic plants of each construct were generated and the presence of the fiber gene was

confirmed. Plants expressing HEV mRNA were further screened using turkey anti-HEV antibody to identify lines expressing high levels of fiber protein.

Fiber protein from these plants will be used in an immunization trial to determine if plant-expressed antigen is capable of introducing protective antibodies against HEV.

The experiments contained in this thesis are the first step in demonstrating the usefulness of transgenic tobacco as edible vaccines to protect against viral diseases in turkeys.

## Chapter II
## Materials and methods

### Hemorrhagic enteritis virus purification from turkey spleen

Turkey spleens (150g) containing HEV-A were homogenized in 200 ml Tris - buffer (20 mM Tris - HCl [pH 7.8], 2 mM $MgCl_2$) using a glass blender at full speed and then sonicated using a Sonic Dismembrator™ (Model 550; Fisher, Pittsburgh, PA) at 100% power for 40 seconds (medium probe; diameter 1 cm). The sonicate was centrifuged at 5,000 x g at 4°C for 25 min; about 200 ml of supernatant was collected. Sodium chloride (5.8 g) and polyethylene glycol 8000 (20 g) were added gradually into the supernatant in succession with constant stirring. The mixture was placed at 4°C for 6 hr and then centrifuged at 5,000 x g at 4°C for 25 min. The supernatant was loaded onto sucrose / CsCl gradients set up in 25 x 89 mm tubes (Beckman, Palo Alto, CA) by adding consecutively 10 ml 40% CsCl, 10 ml 35% CsCl, 6.5 ml 1.1M sucrose and 8 ml turkey spleen supernatant and overlaid with mineral oil. Gradients were centrifuged at 100,000 x g at 4°C for 23 hr in a SW27 rotor (Beckman, Polo Alto, CA). The virus band was collected with an 18 guage needle. The virus suspension was dialyzed 3 times for 12 hr in 500 ml dialysis buffer (2 mM Tris - HCl [pH 7.8], 2 mM $MgCl_2$) at 4°C, lyophilized overnight and then resuspended in 1 ml of 20 mM Tris - HCl (pH 7.8).

### Analysis of purified HEV by SDS-PAGE and western blotting

Purified HEV virus was fractionated on 12.5% denaturing polyacrylamide gels at 25 mA / gel for one hour and a half at 85 volts. For detection of total protein, gels were soaked in Coomassie blue dye for 4 hr at room temperature and then destained in 15% methanol, 10% acetic acid for at least 8 hr at room temperature. The gel was rinsed with distilled water and dried at room temperature using a gel dryer (Bio - Rad, Hercules, CA). For immuno detection, gels wer transferred to Optitran® nitrocellulose membranes (Schleicher & Schuell, Keene, NH; 0.45 μm pore size). The membranes were blocked with sufficient assay buffer (PBS [pH 7.0], 0.1% Tween - 20, 5% dry milk) to cover the whole membrane at 4°C overnight. Membranes were incubated in succession with absorbed turkey anti - HEV sera (provided by Dr. F. W. Pierson, Virginia Tech) diluted 1:500 in assay buffer and secondary antibody mouse anti - turkey IgG - alkaline phosphatase conjugate (Kirkegaard and Perry Laboratories, Gaithersburg, MD) diluted 1:3000 in assay buffer. After antibody incubations, membranes were washed four times for 15 min with PBS (pH 7.0) containing 0.1% Tween 20 and equilibrated by detection buffer (0.1M Tris - HCl [pH 9.5]) for 2 min. Membranes were then incubated with 10 μl / ml CDP star (Boehringer Mannheim, Indianapolis, IN) and 50 μl / ml Enhancer (Boehringer Mannheim, Indianapolis, IN) diluted in detection buffer for 5 min at room temperature. Blots were exposed to Kodak XAR film for 1 to 5 min at room temperature.

### Fiber protein purification from *E. coli* by affinity chromatography

The fiber gene was cloned in the *NcoI / SpeI* site of the multiple cloning site of plasmid pRSETB (provided by S. M. Boyle, Virginia Tech), which provides N-terminal T7-tag (10 amino acids) and polyhistadine tag upstream of the multiple cloning site. A 500 ml culture of *E. coli* containing plasmid pRSETB (Invitrogen, Carlsbad, CA) bearing a fiber gene was grown in LB with ampicillin (100 μg / ml) at 37°C with vigorous shaking to an $OD_{550}$ = 0.6. IPTG (Isopropyl - β - D - thiogalactopyranoside) was added

into *E. coli* culture to 1mM for induction of the fiber gene expression. The *E. coli* was cultured at 37°C with vigorous shaking for another 3 hr and collected by centrifugation at 5,000 x g for 25 min and resuspended in 15 ml of binding buffer (20 mM sodium phosphate [pH 7.8], 500 mM NaCl); cells were lysed by passing through a French Pressure cell at 20,000 psi (pounds per square inch) three times. Insoluble cell debris was removed by centrifugation at 3000 x g for 15 min at 4°C. The supernatant was clarified by passing through a 0.8 μm syringe filter. About 5 ml of ProBond Resin (Invitrogen, Carlsbad, CA) was equilibrated with same volume of binding buffer and then mixed with *E. coli* lysate at 4°C for 2 hr. The mixture was poured into a column (2.5cm x 10cm) in a vertical position and the resin was allowed to settle by gravity. The column was washed in succession by binding buffer and washing buffer (20 mM sodium phosphate [pH 6.0], 500 mM NaCl) until the $OD_{280}$ of the flow through was less than 0.01. The fiber protein was eluted by applying consecutively 5 ml of three imidazole elution buffers in increasing imidazole concentration (i.e. 50 mM, 200 mM, and 500 mM). All flow-through, washes and elutes were collected, aliquoted and stored at -80°C.

**Hyperimmune serum absorption by *E. coli* total protein**

Crude *E. coli* extracts were used to reduce non-specific binding of the anti-HEV turkey serum. *E. coli* strain DH5α with only pRSETB plasmid was cultured overnight in LB with ampicillin (100 μg / ml). *E. coli* cells were harvested by centrifugation at 5000 x g for 15 min at 4°C. The cell pellet was resuspended in PBS (pH 7.0) and lysed by passing through a French Pressure cell at 20,000 psi for three times. The turkey - anti - HEV serum was diluted 1:500 in PBS and mixed with cell lysate (5ml lysate / 500 ml diluted serum). The mixture was rotated on a shaker for 1 hr at room temperature. Another 5 ml of lysate can be added and incubated with serum for 1 hr at room temperature. The mixture was centrifuged at 100,000 x g for 1 hr at 4°C. The supernatant was sterilized by passing through a 0.45 μm membrane and stored at 4°C in aliquots.

**Analysis of purified fiber protein by western blot analysis**

Eluates from the nickle affinity column (Invitrogen, Carlsbad, CA) were fractionated on 12.5% denaturing polyacrylamide gels at 25 mA / gel for 1 hr and 40 min and then transferred to nitrocellulose membranes using a semidry blotter (Bio - Rad, Hercules, CA) at 0.07 v / gel for 1 hr and 30 min. Membranes were blocked with enough blocking buffer (PBS [pH 7.0], 2% BSA) to cover the whole membrane for 1 hr at room temperature. Membranes were then incubated in succession with mouse anti - T7 serum diluted 1:10,000 in PBS, and goat anti - mouse IgG -horseradish peroxidase conjugate (Kirkegaard & Perry Laboratories, Gaithersburg, MD) diluted 1:800 in PBS. Bound antibody was detected with 4 - chloro - 1 - naphthol reagent (Sigma, St. Louis, MO).

Eluates from Nickle affinity chromatography were also tested by western blot analysis using absorbed turkey - anti - HEV serum diluted 1:500 in PBS as primary antibody, and goat - anti - turkey IgG - alkaline phosphatase conjugate (Kirkegaard & Perry Laboratories, Gaithersburg, MD) diluted 1:800 in PBS as secondary antibody. Protein bands were detected with BCIP (5-bromo-4-chloro-3-indolyl phosphate disodium) / NBT (nitro blue tetrazolium chloride) substrate (Bio-Rad, Hercules, CA).

**Polyclonal antibody production using fiber protein purified from recombinant *E. coli***

A ten-week-old rabbit was immunized four times at 2-week intervals with 100 μg of fiber protein purified from recombinant *E. coli*. The fiber protein was suspended in PBS (pH 7.2) and emulsified in Freund's complete adjuvant (volume 1:1) and injected intramuscularly for the primary immunization. In the subsequent three injections, 100 μg of fiber protein was emulsified in Freund's incomplete adjuvant (volume 1:1) and injected as described above. Blood samples were drawn from the marginal ear vein before each injection; after the last booster, blood was collected from the rabbit heart to yield 70 ml anti – fiber serum.

**Western blot analysis for rabbit serum**

Purified fiber protein from recombinant *E. coli* was separated by 12.5% denaturing acrylamide gel electrophoresis, transferred to nitrocellulose membranes and blocked as described before. The membrane was incubated in succession with rabbit anti-fiber serum diluted 1:200 in PBS (pH 7.0), and goat anti-rabbit IgG - horseradish peroxidase conjugate (Kirkegaard & Perry Laboratories, Gaithersburg, MD) diluted 1:800 in PBS (pH 7.0). Bound antibodies were detected with 4–chloro–1–naphthol reagent (Sigma, St. Louis, MO). Tobacco total protein extracted from transformant 35S-41 was also separated by denaturing acrylamide gel electrophoresis, transferred to nitrocellulose membrane, blocked and processed with the rabbit anti-fiber serum as described above.

**Turkeys for protection test**

Commercial, large white turkey hens were obtained from a local hatchery (British United Turkeys of America, Lewisburg, WV). They were raised in batteries until 3 weeks of age, at which time they were moved to floor pens with pine shaving litter. Food and water was provided *ad libitum.* At 6 weeks of age the birds were bled to assure the absence of antibodies to HEV.

**Fiber protein immunization and challenge.**

Six-week-old turkeys, housed in isolation units, were immunized twice at 2-week intervals. Fifteen turkeys were divided into three groups of 5 birds. Fiber protein produced by recombinant *E. coli* was purified by affinity chromatography as described above and injected into turkeys as described below.

The first injection, containing 100 μg fiber protein emulsified in Freund's Complete Adjuvant (volume 1:1), and the second injection containing 100 μg fiber protein emulsified in Freund's Incomplete Adjuvant (volume 1:1), were given as intramuscular injections in the chest region of birds at interval of 15 days. Purified HEV-A ( approximatly 100 μg fiber protein / dose) suspended in PBS (pH 7.0) and PBS (pH 7.0) alone were used as positive and negative controls, respectively. Immunized and control birds were challenged orally with 100 $TID_{50}$ (median turkey infectious dose) of HEV-A 7 days after the second immunization. Blood samples for serum analysis were taken weekly, starting from the first immunization to the time the birds were killed. Birds were killed by cervical dislocation 5 days after challenge and then were necropsied and spleens were collected for HEV analysis.

**Immuno-double-diffusion analysis of turkey spleen and serum**

Agar gels (1%) in 0.5x TBE (45 mM Tris-borate, 1 mM EDTA, pH 7.0) were poured onto slides and allowed to set. Wells (0.5 cm in radius) were punched in the gel and 200 µl of test solutions of antigen (Ag) and antibodies (Ab) were added into wells. Turkey sera and spleen homogenates were tested in three groups. In the first group, HEV virus (Ag) was added in the middle well and turkey serum from different groups were added to surrounding wells. In the second group, fiber protein (about 100 µg for each well) purified from recombinant *E. coli* was placed in the middle well and turkey sera from different groups were added to surrounding wells. In the third group, turkey - anti - HEV sera was placed in the middle well and turkey spleen homogenates from treatment group were added to surrounding wells. Agar gel slides were incubated at room temperature in the dark for 48 hr to allow the formation of precipitation lines.

**Western blot analysis for turkey serum**

Fiber protein purified from recombinant *E. coli* was separated by denaturing acrylamide gel electrophoresis, transferred to nitrocellulose membranes and blocked as described before. The membrane was incubated in succession with the turkey serum of the treatment group diluted 1:50 in PBS and goat anti – turkey IgG - alkaline phosphatase conjugate diluted 1:500 in PBS. Bound antibodies were detected by BCIP / NBT substrate as described before.

**Construction of plant vectors for plant transformation.**

A plasmid called pHE302 (provided by Dr. S. M. Boyle) contains the full length of HEV fiber gene (1.36 kb, Genbank accession number NC 001958 ). The HEV fiber gene was excised from pHE302 by *NcoI* and *XbaI* restriction digestion by Kelly S. Moffat (Dr. C. L. Cramer's lab). The vector called pRTL2 (provided to Dr. C. L. Cramer's lab by Dr. J. Mollet, Texas A&M) contains the double enhanced 35S promoter and the TEV translational enhancer (Carrington & Freed, 1990). The vector was digested by *NcoI* and *XbaI* and ligated with HEV fiber fragment. The whole plasmid (pKM1) was then transformed into *E. coli* strain DH5α. The CaMV 35S : fiber fragment was purified as a HindIII – XbaI fragment and subcloned into *HindIII / XbaI* digested pBIB-HYG (Becker, 1990) to yield pYT-1 (see Fig. 2). At the same time, the 1.4kb fiber gene was excised by *NcoI / XbaI* from pKM1, and a 0.5kb promoter named MeGA was obtained by *HindIII / NcoI* digestion of pCT 155 (CropTech Corporation, Blacksburg, VA). The MeGA promoter is wound-inducible and has been modified from a defense - related tomato *hmg2* gene so that it is generally inactive during normal growth, but shows strong activation in response to mechanical stress such as wounding (Cramer *et al.,* 1990). The MeGA promoter and HEV fiber gene were subcloned into the *HindIII / XbaI* digested pBIB-HYG (see Fig. 2) and named pYT-2. The promoter: fiber constructs lie between the T-DNA left border and right border of pBIB-HYG and limits the DNA that is integrated into plant genome. The nopaline synthase terminator is down stream of promoter:fiber region; there is a hygromycin phosphotransferase gene upstream that allows selection of transgenic plants with hygromycin. Both pYT-1 and pYT-2 were transformed individually into *E. coli* DH5α competent cells (Life Technologies, Gaithersburg, MD) by a heat-shock method (Cohen *et al.,* 1972). pYT-1 and pYT-2 were isolated from *E. coli* and transformed into *Agrobacterium tumefaciens* strain LBA4404 by the freeze-and-

thaw method (An, G., 1987). The cloned inserts in transformed *Agrobacterium* LBA4404 strains were verified by restriction digestions and sizing of fragments by agarose gel electrophoresis.

**Generation of transgenic tobacco**

Tobacco (*Nicotiana tabacum* cv. Xanthi) was transformed by modified leaf-disc cocultivation methods (Horsch *et al.*, 1988) using *Agrobacterium* strains transformed with pYT-1 or pYT-2. After three weeks, shoots were generated from transformed callus selected on regeneration medium (4.3 g / L Murashige and Skoog (MS) salts (Sigma, St. Louis, MO), 0.1% MS vitamins, 100 mg / L myo-inositol, 20 g / L sucrose, 2 mg / L zeatin, 0.5 g/ L carbenicilin, 0.1 g / L hygromycin). Shoots were transferred to selective rooting medium (4.3 g / L MS salts, 0.1% MS vitamins, 30 g / L sucrose, 0.4 g / L $MgSO_4 \cdot 7H_2O$, 0.2 g / L carbenicilin, 0.1 g / L hygromycin) for about two weeks. After healthy roots were formed, plants were transplanted to soil and grown in a plant room at 12 hr sun light conditions.

Transgenic seeds from selected tobacco plants was tested for segregation hygromycin resistance. Seeds were surface sterized by 10% colorox solution and plated (about 25 seeds / plate) on MS medium containing 0.1 g / L hygromycin. After 3-4 weeks, sensitive and resistant seedlings were counted.

**Tobacco genomic DNA extraction and PCR**

Tobacco leaves (8 gram) were frozen in liquid nitrogen and ground to a fine powder using a pestle and motor; the powder was resuspended in 16 ml CETAB buffer (1% hexodecyltrimelthyl ammonium, 50mM Tris - HCl [pH 8.0], 10mM EDTA, 0.7M NaCl, 1% N-lauroylsarcosine). One volume of phenol (purity > 99.0%) was added, mixed well, and the mixture was centrifuged at 15,000 x g for 10 min. The supernatant was mixed with one volume of phenol / chloroform / isoamylalcohol (25:24:1) and centrifuged at 15,000 x g for 10 min. The supernatant was collected and 1/10 volume of 3M NaOAc and 2 volumes of 100% ethanol were added and mixed well. The mixture was placed in -20°C for 30 min and centrifuged at 12,000 x g for 10 min. The DNA pellet was dried at room temperature and resuspended in 400 µl TE buffer (10mM Tris-HCl [pH 8.0], 1mM EDTA [pH 8.0]) at final concentration of 2.5 µg/µl. Ten µl RNAase A (10 unit / µl; Life Technologies, Gaithersburg, MD) was added into the DNA / TE buffer and incubated overnight to eliminate RNA contamination.

Purified tobacco genomic DNA was diluted 1 : 100 and was used as template for PCR reaction using PCR Ready ToGo Beads (Pharmacia, Piscataway, NJ). Specifically designed fiber primers (Genosys, The Woodlands, TX) RTHEVF (5'-CATTTATTGCCCATGGCTACTCC; 1 nmol / µl), and RTHEV3 (5'-AACACTAACTGCTAGACCATTT; 1 nmol / µl) were diluted 1 : 50 and mixed at ratio of 1:1. The total 25 µl PCR reaction consisted of 1 µl tobacco genomic DNA (25 ng / µl), 1 µl primer mixture (0.02 n mol / µl) and 23 µl $H_2O$. The reaction cycle was 94°C, 5 min for one cycle; 94°C, 1.5 min, 54°C, 1 min, 72°C, 1.5 min for 35 cycles; 72°C, 10 min for one cycle. The PCR products were size-seperated on 0.8% agarose gels and the product bands were revealed by staining the gel with ethidium bromide and visualized by UV

light; they were sized by comparison to molecular weight DNA markers.

**RNA extraction and hybridization**

About 2 grams of leaves from transgenic tobacco, transformed with MeGA : fiber construct, were wound-induced by slicing in about 1 mm strips and incubated at room temperature for 12 hours. Total RNA was then isolated by RNeasy Plant Mini Kit (Qiagen, Valencia, CA) from the treated MeGA tobacco leaves as well as from young tobacco leaves containing 35S promoter : fiber construct. Samples of 20 μg total RNA were denatured with 17.5% formaldehyde and 50% formamide in MOPS buffer (20 mM 3-N-Morpholino proponesulfonic acid [pH 7.0], 8 mM sodium acetate, 1 mM EDTA [pH 8.0]), fractionated on 1% agarose gels with MOPS buffer. The RNA was blotted to nylon membranes (0.45 μm pore size) and UV cross-linked under 254 nm UV irradiation. The membranes were hybridized to a digoxigenin (DIG; Boehringer Mannheim, Indianapolis, IN) -labeled DNA probe amplified from the 1.4 kb *NcoI / XbaI* fiber fragment of pKM1 by PCR using fiber primers RTHEVF and RTHEV3 (see above). Blots were hybridized at 44°C in DIG Easy hybridization buffer (Boehringer Mannheim, Indianapolis, IN) overnight. Blots were then washed at room temperature twice with 2xSSC (0.3 M NaCl, 0.03 M sodium citrate [pH 7.0]) containing 0.1% SDS and another two times with 0.1xSSC containing 0.1% SDS at 68°C, and then blocked with blocking reagent (Boehringer Mannheim, Indianapolis, IN) for 30 min at room temperature. DIG-alkaline phosphatase antibody conjugate was added to the membrane and incubated for 30 min at room temperature with gentle agitation and then washed with washing buffer (0.1 M maleic acid [pH 7.5], 0.15 M NaCl, 3% Tween 20) for 5 min. The blot was equilibrated with detection buffer (0.1 M Tris - HCl, 0.1 M NaCl, 50 mM $MgCl_2$) for 2 min and incubated with chemiluminescent substrate CDP star (Beohringer Mannheim, Indianapolis, IN) diluted in detection buffer at 50 μl / ml for 5 min. The blot was then exposed to Kodak XAR film for 1 min at room temperature.

**Screening of transgenic tobacco plants by RNA dot blot analysis**

Leaves from individual transgenic tobacco (2 grams for each plant) containing MeGA promoter were wounded as described before. Total RNA (20 μg) from tobacco leaves expressed from 35S promoter or the MeGA promoter were isolated by RNeasy Plant Mini Kit (Quaigen, Valencia, CA) and dot blotted by a dot-blot apparatus on positively charged Nytran$^{+®}$ nylon membranes (Schleicher & Schuell, Keene, NH; pore size 0.45 μm). Blots were hybridized and developed as described above. Two tobacco lines expressing RNA using the 35S promoter (35S-25, 35S-41) and two tobacco lines using MeGA promoter (MeGA-7, MeGA-9) were selected for further experiments.

**Protein extraction from selected transgenic tobacco**

Transgenic tobacco leaves containing MeGA promoter were wounded by cutting and incubated at room temperature for times of: 0hr, 8hr, 24hr, and 48hr. At each time point, 10 grams of leaves were placed into -80°C freezer for storage. Seeds of transgenic tobacco containing 35S promoter were planted. After approximately four weeks, seedlings, about 10 cm high, were harvested. Tobacco leaves were homogenized (1 g tissue / 2 ml buffer) in extraction buffer (0.1 M potassium - phosphate [pH 7.0], 5 mM dithiothreitol) by a Ten-Broek$^{TM}$ tissue grinder with clearance 0.152 mm (VWR,

Bridgeport, NJ) and then centrifuged at 10,000 x g, 4°C for 30 min. The supernatant was centrifuged at 20,000 x g, 4°C for 30min and then concentrated overnight by 10 kDa cut off Centricon filter (Millipore Corporation, Bedford, MA). Total protein concentration was measured by a Coomassie dye-binding assay (Bio-Rad, Hercules, CA) using BSA as a standard. Total protein from 35S-41 transgenic tobacco seeds was also extracted and measured as described above.

**Hyperimmune serum absorption by nontransgenic tobacco total protein**

In order to reduce non-specific binding of turkay antiserum to tobacco protein, the serum was preabsorbed with tobacco extracts. Total protein from nontransgenic tobacco (10 g) was extracted by grinding leaves in liquid nitrogen and resuspending in extraction buffer as described above at a final concentration of 2.5 μg / μl. The turkey anti - HEV serum (provided by F. W. Pierson, Virginia Tech) was diluted 1:50 in PBS and mixed with total tobacco protein (5 ml tobacco protein / 200 ml diluted serum). The mixture was shaken gently for 2 hr at room temperature and another 5 ml tobacco protein was added and shaken for 2 hr at room temperature. The mixture was centrifuged at 100,000 x g for 1 hr at 4°C in a SW27 rotor (Beckman, Palo Alto, CA). The supernatant was sterilized by filtration through a 0.45 μm membrane, aliquoted and stored at -20°C.

**SDS / PAGE and western blot analysis of total protein from transgenic tobacco leaf and seed**

Concentrated transgenic tobacco leaf protein samples were fractionated on 12.5% denaturing polyacrylamide gels at 25 mA / gel for one hour and 40 min (Frederick *et al.*, 1989) and then transferred to Optitran® nitrocellulose membranes (Schleicher & Schuell, Keene, NH; 0.45 μm pore size) using a semi - dry blotter (Bio-Rad, Hercules, CA) at 0.07 V / gel for one hour and 30 min. Concentrated transgenic tobacco seed protein samples were also fractionated on polyacrylamide gel and was transferred to Optitran® nitrocellulose membranes as described above. The membranes were blocked with sufficient assay buffer (PBS [pH 7.0], 0.1% Tween - 20, 5% dry milk) to cover the whole membrane at 4°C overnight. Membranes were incubated in succession with absorbed turkey anti - HEV sera (provided by Dr. F. W. Pierson, Virginia Tech) diluted 1:500 in assay buffer and secondary antibody mouse anti - turkey IgG - alkaline phosphatase conjugate (Kirkegaard and Perry Laboratories, Gaithersburg, MD) diluted 1:3000 in assay buffer. After antibody incubations, membranes were washed four times for 15 min with PBS (pH 7.0) containing 0.1% Tween 20 and equilibrated by detection buffer (0.1M Tris - HCl [pH 9.5]) for 2 min. Membranes were then incubated with 10 μl / ml CDP star (Boehringer Mannheim, Indianapolis, IN) and 50 μl / ml Enhancer (Boehringer Mannheim, Indianapolis, IN) diluted in detection buffer for 5 min at room temperature. Blots were exposed to Kodak XAR film for 1 to 5 min at room temperature.

**Sandwich ELISA**

Fiber protein expressed by tobacco leaf was quantified by sandwich ELISA. Turkey anti - HEV serum (diluted 1:4000 in PBS [pH 7.4]) was bound to 96-well polyvinylchloride microtiter plates (Nunc-Immuno™ plate with MaxiSorp™ surface) at 100 μl / well at 4°C overnight. The plate was blocked with assay buffer (PBS [pH 7.4], 2% bovine serum albumin) at 200 μl / well for 1 hr at room temperature. After washing

the wells five times with 50 mM Tris - HCl, 0.2% Tween 20 (500 µl / well), tobacco protein samples diluted 1:100 in assay buffer were added (100 µl / well) and incubated 3 hr at room temperature; each sample was tested in duplicates. After a second wash as described above, mouse-anti-HEV fiber monoclonal sera diluted 1:100 in assay buffer was added (100 µl / well) and incubated 1 hr at room temperature. The wells were washed and incubated with 100 µl / well biotinylated goat-anti-mouse sera (Caltag Laboratories, San Francisco, CA) diluted in assay buffer (0.25 µg / ml) for 1 hr at room temperature. The wells were then incubated with 100 µl / well horseradish peroxidase - streptavidin (Caltag Laboratories, San Francisco, CA) diluted 1:5000 in assay buffer with 0.05% Tween - 20 for 30 min in dark at room temperature. After another wash, the plate was developed with 100 µl / well of TMB peroxidase substrate (Kirkegaard and Perry Laboratories, Gaithersburg, MD) for 30 min in the dark at room temperature. The reaction was stopped by adding 100 µl / well of 0.185 M sulfuric acid and the absorbance read at 450 nm using a microplate reader (Molecular Devices, Sunnyvale, CA). For a standard curve, HEV purified from infected turkey spleen by precipitation and sucrose / CsCl gradient centrifugation as described below was diluted in PBS (pH 7.0) to a concentration of 50 ng / ml and processed as above.

**Fiber protein purification from tobacco leaves by immuno-affinity column chromatography**

Turkey anti - HEV serum was absorbed by nontransgenic total tobacco protein as described before. The serum was diluted 1:50 in PBS (pH 7.0). One gram of CNBr - activated Sepharose 4 Fast Flow Resin® (Pharmacia, Piscataway, NJ) was washed with 10-15 volumes of cold 1 mM HCl 6 times to wash out sugar additives on the surface of resin. About 12 ml of diluted (1:50) turkey serum was mixed with 2 ml of washed resin and incubated at room temperature for 4 hr with constant shaking for complete coupling. Unreacted ligand was blocked by mixing with 12 ml of 0.1 M Tris - HCl (pH 8.0) for 5 hr at room temperature. The coupled resin was washed alternately with 10 ml of low pH buffer (0.1 M NaOAc, 0.5 M NaCl, pH 4.0) and 10 ml of high pH buffer (0.1 M Tris - HCl, 0.5 M NaCl, pH 8.0) 6 times. Then the resin was washed by 12 ml of protein extraction buffer (0.1M potasium phosphate [pH 7.0], 5 mM DTT) twice and then mixed with tobacco total protein extract at 4°C overnight. The mixture was poured into a column (2.5 cm x 10 cm) in a vertical position to allow the resin to settle completely by gravity. The fiber protein was eluted by adding 0.7 ml of 0.1M glycine (pH 2.8) to the column. Tris - HCl (pH 8.0) was added into the eluate to adjust pH to 7.0. The eluate was concentrated using a 10 kDa cut off Centricon filter (Millipore Corporation, Bedford, MA) overnight. The concentrated eluate was analyzed by Western blotting using absorbed turkey anti - HEV serum and goat anti - turkey IgG - alkaline phosphatase conjugate. The membranes were developed by CDP star substrate (Boehringer Mannheim, Indianapolis, IN) as described before.

**Rubisco crystallization**

Total tobacco protein was extracted from transformant 35S-41 as described before. The extract was divided into four groups and centrifuged at 12,000 x g at 4°C for 20 min. The pH of each supernatant was adjusted using acetic acid to pH 4.0, 4.5, 5.0, and 5.5, respectively. The extractions were placed at 4°C overnight to allow ribulose bisphosphate

carboxylase (Rubisco) to precipitate and then centrifuged at 12,000 x g at 4°C for 20 min. The supernatant was concentrated by 10 kDa cut off Centricon filter® (Millipore Corporation, Bedford, MA) and analyzed by western blotting as described above.

# Chapter III
# Results

## Fiber protein purification from recombinant *E. coli* by nickle affinity chromatography

Using a prokaryotic system such as *E. coli* to produce recombinant protein is one of the fast and cost-effective ways to make potential recombinant vaccine. It was of interest to know if the *E. coli* - produced fiber protein can induce protection of turkeys to an HEV challenge. The fiber protein, fused with one T7-tag and one his-tag, was purified from *E. coli* using nickle affinity chromatography. All flow-through, washes and eluates from the nickle affinity chromatography were analyzed by western blotting using the mouse anti-T7 tag sera. The western blotting (see Fig. 2) shows that the fiber protein (about 50 kDa) was in the fraction eluted by 200 mM imidazole. Minor bands were observed at various molecular weights and probably represent degradation of the 50 kDa fiber protein. No signal was detected in fractions of flow-through and washes (data not shown). Although the natural fiber protein in HEV capsid is 29 kDa, the fiber protein expeced from *E. coli* is about 50 kDa. When using the turkey anti-HEV serum as the first antibody to react with eluants of 200 mM imidazole, there was clearly a band at 50 kDa (see Fig. 3). The results demonstrated that the fiber protein produced by *E. coli* was recognized by anti-HEV serum raised in turkeys.

## Polyclonal antibody production

The fiber protein was purified from recombinant *E. coli* and injected into a rabbit four times in order to produce specific anti-fiber serum. When the fiber protein was purified by a nickle affinity chromatography, nonspecific *E. coli* proteins, eluted with the fiber protein. After the rabbit was injected with the fiber plus contaminating proteins, it not only produced antibodies to fiber protein, but also to *E. coli* proteins. The western blot (see Fig. 4) showed high background. However, the fiber protein band can still be distinguished from the background and demonstrated that the rabbit produced antibodies specific for fiber. On the blot, the fiber protein co-migrated with the positive control that was detected by using specific anti-T7 tag serum. The rabbit serum was also tested by reacting with transgenic tobacco protein (see Fig. 5). The 49 kDa fiber protein band can be faintly seen on the western blot.

## Virus purification

In order to have a positive control for the turkey vaccine trial, HEV-A was purified from infected turkey spleens by ultra-centrifugation of CsCl gradients. After the ultra-centrifugation, there was clearly one white virus band in the gradient. After dialysis and lyophlization, the virus was resuspended in PBS (pH 7.0) at a final protein concentration of 2.8 μg / μl. The HEV virus was analyzed by SDS - PAGE followed by western blotting (see Fig. 6) in order to differentiate HEV polypeptides and to confirm that the band collected by CsCl gradient was HEV. About nine polypeptides were found in stained SDS-PAGE gel. These bands were also recognized specifically by antibodies from HEV-infected turkeys. According to published data (van den Hurk, 1992), the 96 kDa band is hexon, the 51 kDa band is penton base, and the 29 kDa band is fiber protein.

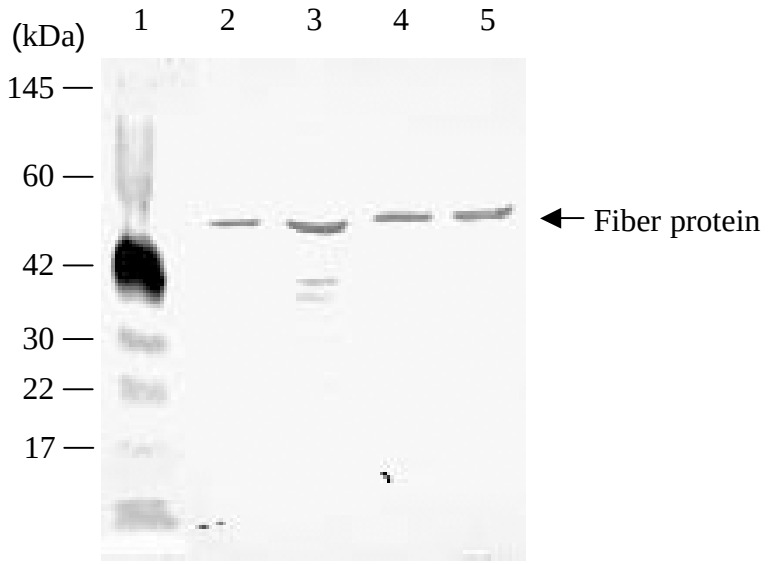



Fig. 2. Immunoblot analysis of fiber protein purified from recombinant *E.coli*. Protein eluted from the nickle affinity chromatography (20 μg per lane) was gel separated and transferred onto the membrane. The recombinant fiber protein was detected by mouse anti - T7 serum. Lane 1: molecular weight marker; Lanes 2 – 5: fiber protein eluted from affinity column.

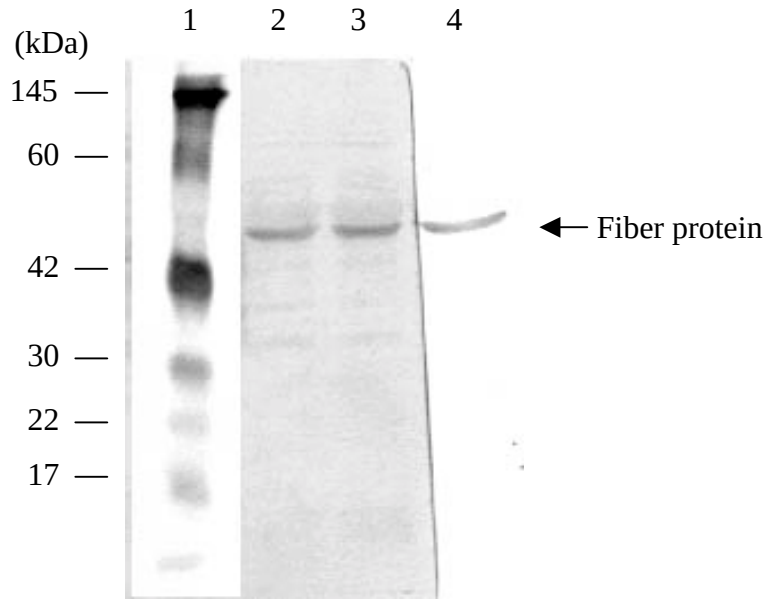



Fig. 3. Immunoblot analysis of fiber protein purified from recombinant *E.coli*. Total protein eluted from nickle affinity column (20 µg per lane) was detected using turkey anti – HEV serum. Lane 1: molecular weight marker; Lanes 2 - 3: fiber protein reacted with turkey anti - HEV sera; Lane 4: fiber protein reacted with mouse anti – T7 serum as positive control.

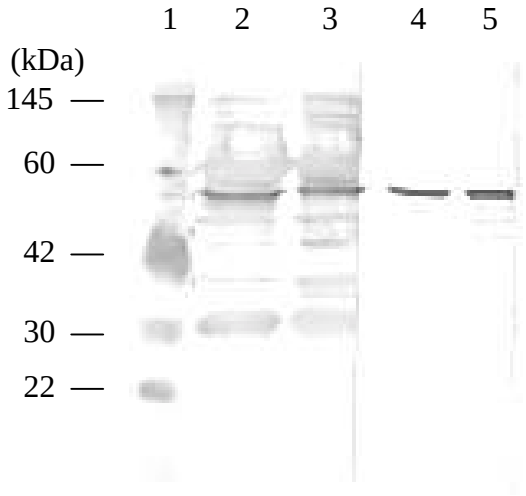



Fig. 4. Immunoblot analysis testing *E. coli*-synthesized fiber cross-reactivity with rabbit-anti–fiber serum. Fiber protein purified from recombinant *E. coli* (20 μg per lane) was blotted on membrane and reacted with rabbit-anti-HEV serum. Lane 1: molecular weight marker; Lanes 2 – 3: fiber protein reacted with rabbit anti – fiber serum; Lanes 4 - 5: fiber protein reacted with mouse anti – T7 serum as positive control.

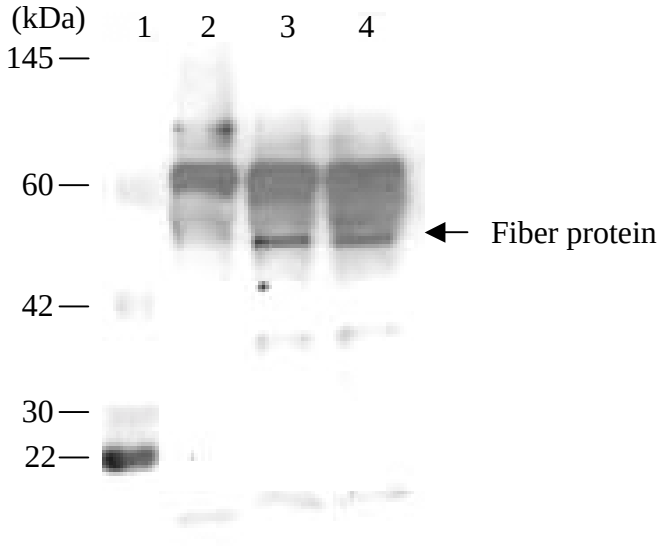



Fig. 5. Immunoblot analysis using rabbit-anti–fiber serum to detect fiber expression in transgenic plants. Total tobacco protein (50 μg per lane) from nontransgenic tobacco (Lane 2) and transgenic tobacco (Lanes 3-4) were blotted on membrane and reacted with rabbit anti-HEV serum. Lane 1: molecular weight marker; Lane 2: nontransgenic tobacco protein (negative control); Lanes 3 - 4: transgenic tobacco (35S – 41) protein.

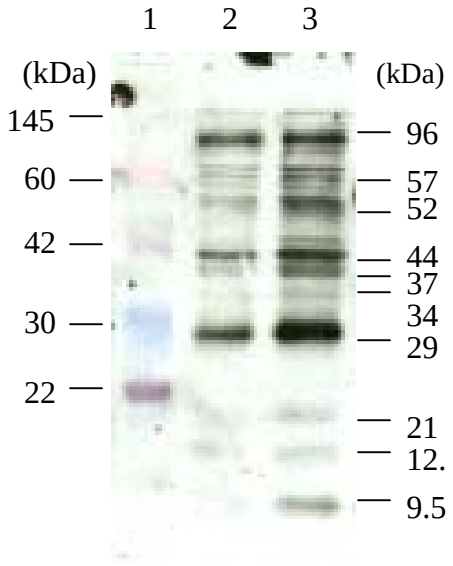



Fig. 6. Immunoblot analysis of HEV purified from infected turkey spleens. HE virus was separated by SDS-PAGE, transferred on to a membrane and reacted with turkey anti-HEV serum. Lane1: molecular weight marker; Lane 2: HEV (10 μg); Lane 3: HEV (30 μg).

**Protection trial**

Turkeys were immunized with *E. coli*-synthesized fiber protein twice at 2-week intervals and were challenged with HEV-A 7 days after the second injection. Turkey spleens were collected 5 days after HEV-A challenge. Except for the positive control group, all turkey spleens were about double the size of normal turkey spleen, a typical symptom of HEV-A infection. If the injected fiber protein provided protection, spleens would be much smaller in size. Turkey spleen homogenates collected from the treatment group were tested for the presence of virus with turkey-anti-HEV serum by immuno-double-diffusion test; precipitation lines were formed on the agar gel. This result indicates that HEV-A virus replicated in turkey spleen. The virus in spleen homogenates and the anti-HEV serum diffused in the gel and formed the antigen-antibody precipitation lines. This result further demonstrates that the *E. coli*-produced HEV fiber protein did not give protection for turkeys against HEV-A challenge. Turkey sera from the treatment group were tested with fiber protein produced by *E. coli* by western blot and immuno-double-diffusion test. Fig. 7 shows that turkey sera collected from treatment groups could recognize *E. coli*-produced fiber protein (50 kDa). For the immuno-double-diffusion test, precipitation lines were formed on agar gel when turkey serum from treatment group and the *E. coli*-produced fiber protein were used for the reaction. However, the turkey serum failed to form any precipitation line against HEV-A. Based on these results, it appears that the *E. coli*-produced fiber protein is conformationally distinct and can not offer protection for turkeys against HEV challenge. Using a eukaryotic system such as transgenic tobacco maybe a better way to produce the fiber protein.

**Generation of expression vectors and selection of transformed plants.**

The plasmid pYT-1 was constructed by inserting the 35S : HEV fiber construct between the *HindIII* / *XbaI* sites in the plant transformation vector pBIB-HYG (see Fig. 8). In this construct, the expression of fiber protein is driven by dual transcriptional enhanced CaMV 35S promoter for constitutive transcription. The dual enhanced 35S promoter (provided by J. Mullet, Texas A&M) was made by duplicating the CaMV 35S enhancer and linking it to the transcriptional enhancer of the TEV 5' – UTR. The enhanced promoter can increase the expression of linked transgenes. The plasmid pYT-2 differs from pYT-1 by using a wound-inducible promoter (MeGA) to replace the 35S promoter. T-DNA left and right borders flank the promoter and fiber coding region as well as a hygromycin resistant gene which allow selection of plant transformants on media containing hygromycin. The insertions of both constructs (35S:fiber and MeGA:fiber) in pYT-1 and pYT-2 were confirmed by restriction digestion (Fig. 9 and Fig. 10)**.** The plasmids were used to transform tobacco by the leaf disc method using *Agrobacterium,* and at least 60 independent hygromycin-resistant transformants were generated for each constructs.

**Confirmation of fiber gene integration in tobacco genome**

The presence of HEV fiber DNA in the genome of putative transgenic plants was confirmed by PCR amplification. Transgenic tobacco genomic DNA was extracted and resuspended in TE buffer at final nucleic acid concentration of about 2.5 μg/μl. Nontransgenic tobacco genomic DNA was used as negative control and the pYT-1 plasmid containing HEV fiber gene was used as positive control. The results (Fig. 11)

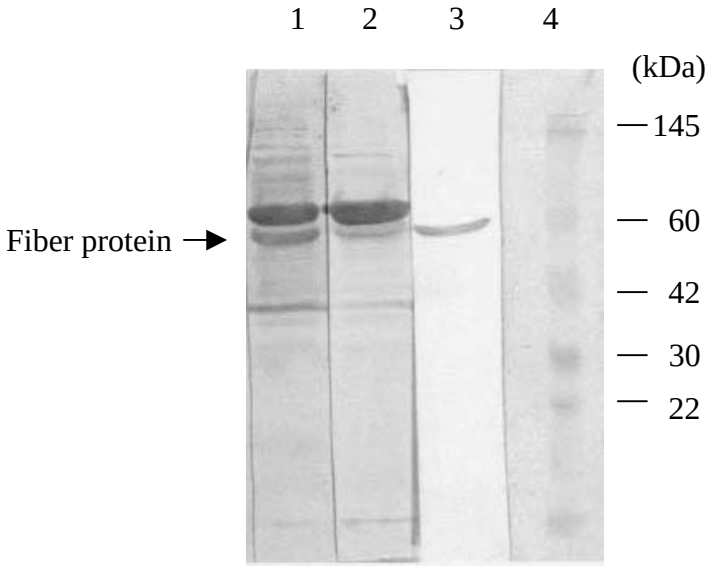
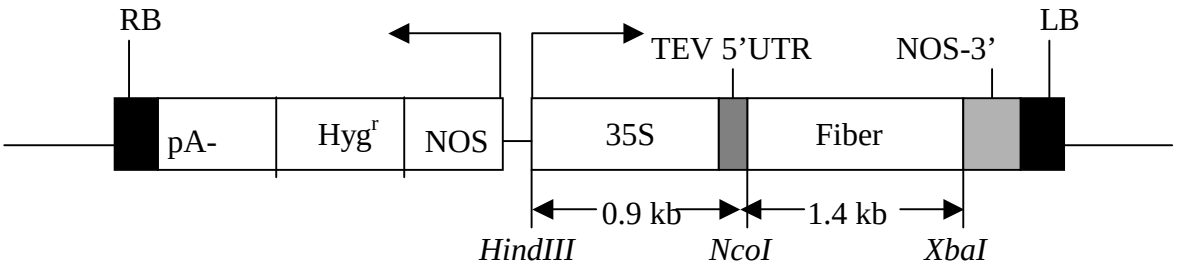
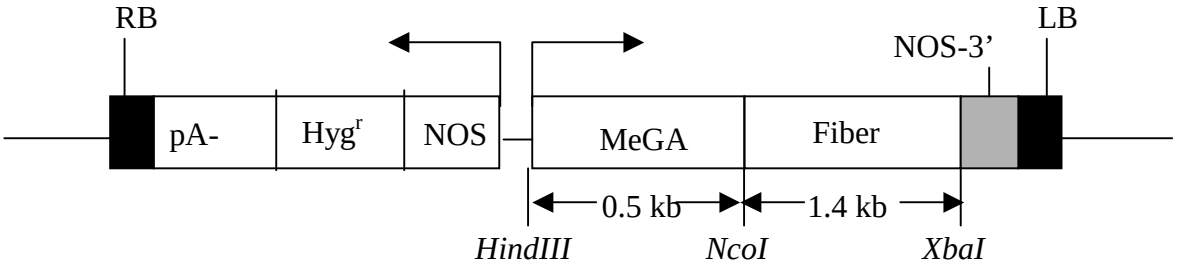



Fig. 7. Immunoblot analysis of anti – fiber serum from turkeys immunized by *E.coli* produced fiber protein. Fiber protein purified from recombinant *E.coli* was gel separated, transferred onto membrane and reacted with turkey anti-fiber serum that was obtained by immunizing turkeys with *E.coli* produced fiber protein. Lane 1 - 2: fiber protein reacted with turkey serum; Lane 3: fiber protein reacted with anti – T7 antibody as a positive control; Lane 4: molecular weight marker.




**pYT-1**




**pYT-2**

Fig. 8. Plasmids used for expression of the HEV fiber sequence in plants. The constructs carry the left and right borders (LB,RB) of the transferred DNA which incorporated into plant genome via *Agrobacterium* – mediated transformation. The fiber coding region lies downstream of the double enhanced CaMV 35S promoter and tobacco etch virus leader sequence (TEV 5'UTR) in pYT-1 and MeGA promoter in pYT-2. Restriction endonuclease cleavage sites are indicated.

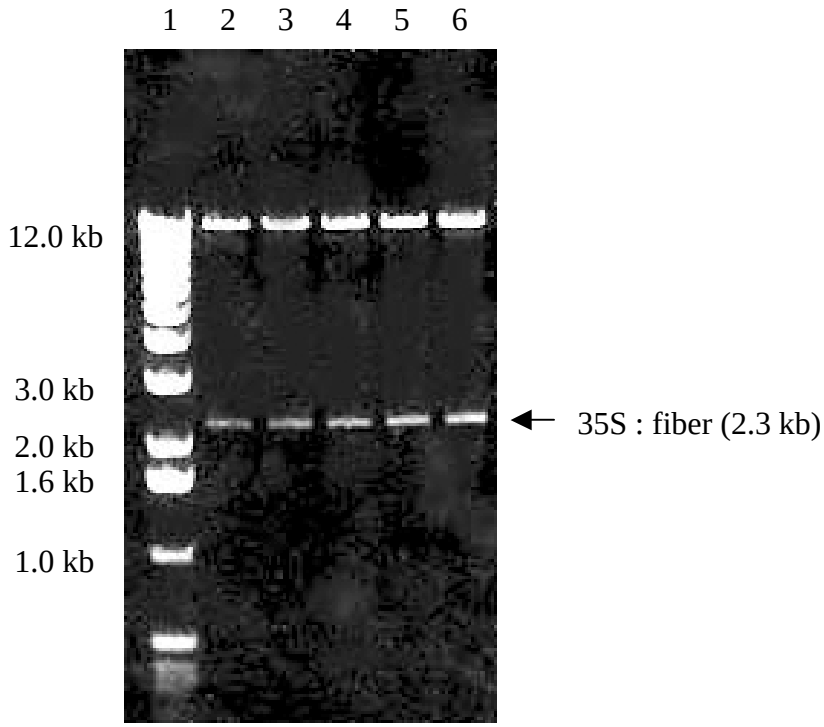



Fig. 9. Restriction endonuclease digestion to confirm insertion of 35S:fiber fragment (2.3 kb) in pBIB-HYG clones. Plasmid DNA from five colonies was digested with HindIII and XbaI and separated in 0.8% agarose in TAE and stained with ethidium bromide. Lane 1: molecular weight standard (1 kb marker); Lanes 2-6: colonies 1-5 respectively.

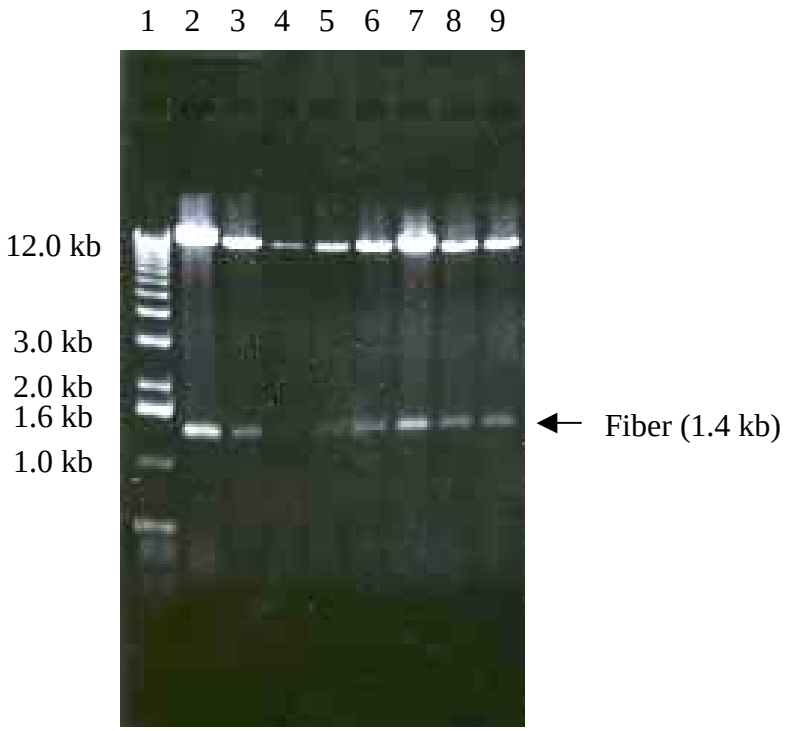



Fig. 10. Restriction endonuclease digestion to confirm insertion of HEV fiber fragment (1.4 kb) in pBIB-HYG. Plasmid DNA from eight colonies was digested with XbaI only and separated in 0.8% agarose in TAE and stained with ethidium bromide. Lane 1: molecular weight standard (1 kb marker); Lanes 2-9: colonies 1-8 respectively.

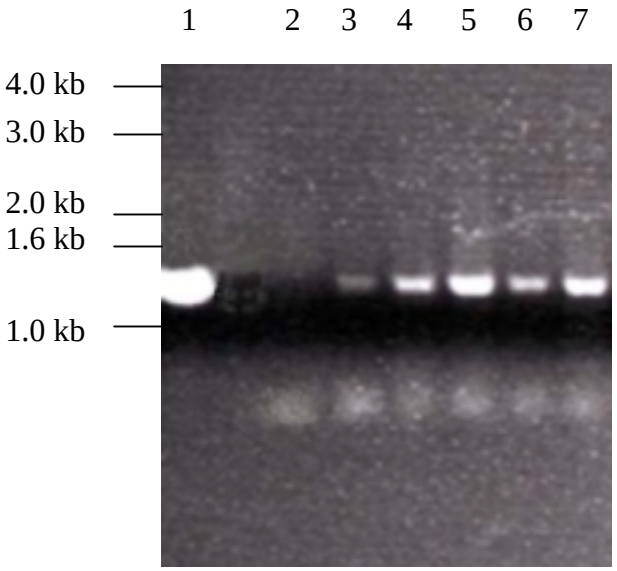



Fig. 11. Detection of the HEV fiber gene by PCR amplification of plant genomic DNA. Same amount of tobacco genomic DNA (25 ng) was used as template for each PCR reaction and same volume of reaction product (10 µl per lane) was loaded in each well of the gel. Lane 1: positive control (plasmid pKM1 containing fiber gene as template); Lane 2: negative control (nontransgenic tobacco genomic DNA as template); Lane 3 - 5: DNA from plants transformed with the 35S:fiber construct; Lane 6 - 7: DNA from plants transformed with the MeGA:fiber construct.

show that a 1.4 kb DNA band appears in each of the transformants. It is consistent with the positive control (lane 1). There is no band from the negative control (lane 2). Signals among tranformants (lanes 3-7) were variable and may reflect different copy number of the fiber gene in the tobacco genome.

Transgene segregation analysis (hygromycin resistance) of seeds from the initial transformat of three 35S:fiber lines (35S-23, 35S-37, 35S-41) suggested a single insert for each line [60R:40S; 66R:39S; 82R:21S for hygromycin resistance ® or sensitivity (S), respectively].

**Transcription of the fiber gene**

Transgenic plants were screened by northern dot blot to identify those with high level of fiber gene mRNA transcription. Because the insertion site of the transferred T-DNA into chromosomal DNA is random and differences in copy number and position effects may occur, a variable level of transcription in independent transformants was expected. In order to identify tobacco plants with higher transcription levels in a short period of time, RNA dot blot analysis were performed. RNA samples were hybridized with a DIG-labeled probe encompassing the coding region of fiber gene. Tobacco leaves containing MeGA promoter and fiber were induced by wounding and incubated at room temperature for 12 hours. Total RNA was extracted from treated MeGA:Fiber tobacco leaves and also from untreated 35S:Fiber tobacco leaves of 5-7 week-old tobacco seedlings. Fig. 12 shows the results of the dot blot of total RNA from about 10 transformants containing MeGA promoter and 20 transformants containing 35S promoter. Total RNA from different nontransgenic tobacco was used as negative control and HEV fiber gene was used as a positive control. Signals among transformants were variable and there was no signal in negative controls. To ensure effective comparisons among transgenic lines, leaves of similar age and size (about 1 inch) were selected for RNA isolation. RNA isolated from older leaves (> 6 inches) of 35S:fiber lines showed significantly less expression (data not shown). Two transformants containing MeGA and two transformants containing 35S (boxed in Fig. 12) were selected from all the transgenic tobacco for further northern blot analysis using the *HindIII* / *XbaI* digested fiber gene as positive control and total RNA from nontransgenic tobacco transformants as negative control. Fig. 13 shows that the mRNA from MeGA and 35S transformants were both about 1.45-1.5kb in length, which is the same size as the positive control. The nontransgenic tobacco leaf RNA showed no detectable signal. Together, these results showed successful integration of the fiber gene into tobacco and mRNA transcription of fiber gene with the correct size in the leaves of selected transformants.

**Hyperimmune serum absorption**

Turkey-anti-HEV serum has a high nonspecific cross-reactivity with total tobacco protein. Fig. 14A shows that almost all the soluble tobacco protein reacted with the turkey serum. Although the serum was diluted to 1:1000, it was still very hard to distinguish a single band from the background. In order to decrease the cross-reaction, total protein from nontransgenic tobacco was mixed with diluted turkey anti-HEV serum and then ultracentrifuged to pellet the protein-antibody combination. Most antibodies that react with nontransgenic tobacco were pelleted and the supernatant was collected for

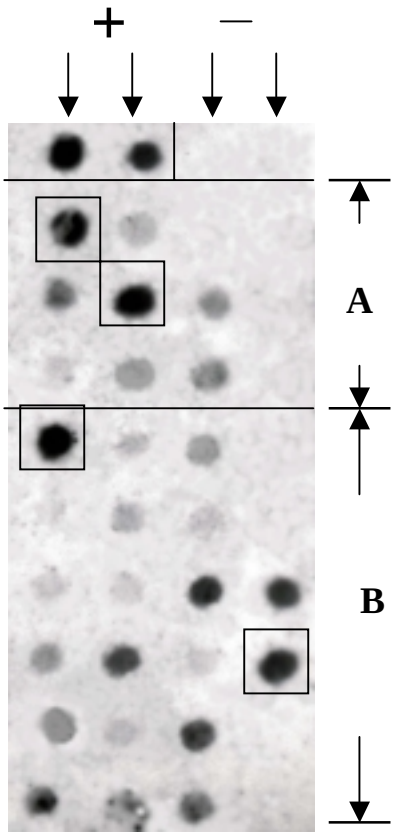



Fig. 12. Dot blot analysis of transgenic tobacco total RNA. The fiber gene (1.4 kb; 2 ng) obtained by *NcoI / XbaI* digestion of pKM1 was used as positive control. Total RNA from nontransgenic tobacco (20 μg) was used as negative control. (A). Total RNA (20 μg / well) from different transformants containing 35S:fiber; (B). Total RNA (20 μg / well) from different transformants containing MeGA:fiber, 12 hr after wound induction. Boxed samples represent those used for northern analysis.

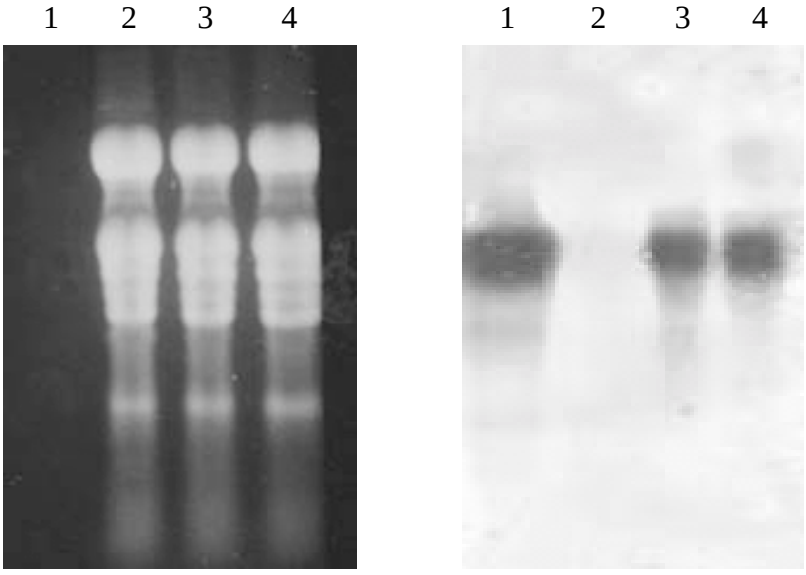



Fig. 13. Northern blot analysis of fiber mRNA transcription in transgenic tobacco. (A). EtBr stained tobacco total RNA. (B). Duplicate northern blot of fiber mRNA. Lane 1, positive control (fiber gene); Lane 2, negative control (total RNA from nontransgenic tobaccol); Lane 3, total RNA from transformant 35S-41; Lane 4, total RNA from transformant MeGA-9, 12 hr after wounding.

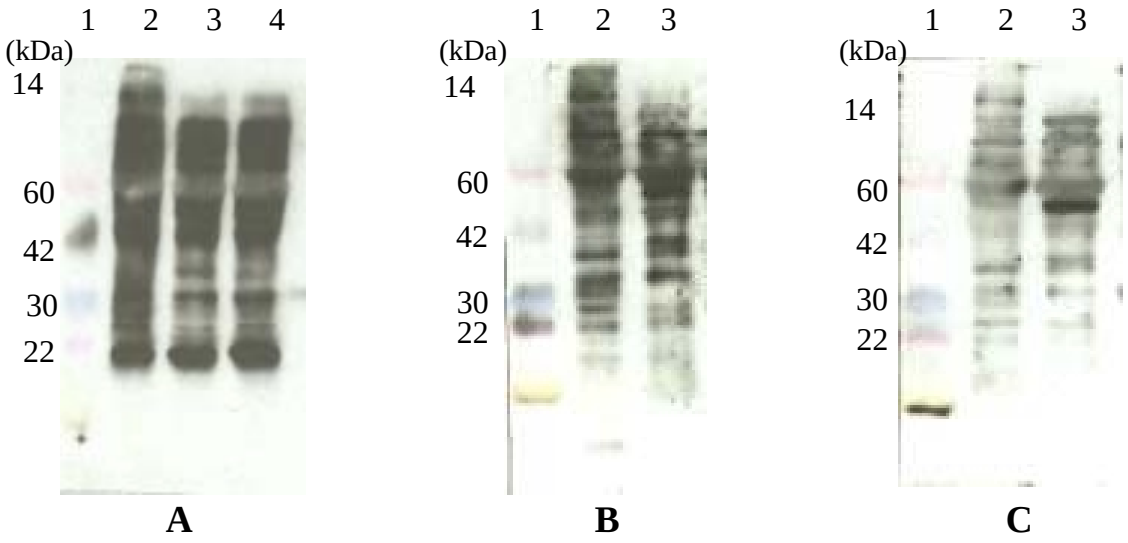



Fig. 14. The effect of serum absorption using nontransgenic tobacco total protein. Total tobacco protein (50 μg per lane) from nontransgenic tobacco (Lane 2) and from transgenic tobacco 35S – 41 (Lane 3,4) were blotted on three membranes in the same pattern. Membranes were reacted with **(A)** nonabsorbed turkey anti – HEV serum diluted 1 : 1000 in PBS; **(B)** absorbed turkey anti – HEV serum diluted 1 : 200 in PBS; **(C)** absorbed turkey anti – HEV serum diluted 1 : 500 in PBS. Lane 1: Marker; Lane 2: total protein from nontransgenic tobacco (negative control); Lanes 3 – 4: total protein from transgenic tobacco 35S – 41.

further experiments. After the absorption, the blots were much clearer and there was a lot less cross-reactivity to tobacco proteins (see Fig. 14B, 14C). The 49 kDa band can be seen clearly. The concentration of the serum was also very important for clear visualization. Compared to the result of using 1:200 diluted serum (see Fig. 14B), the blot using 1:500 dilution of serum allows visualization of the 49 kDa band (see arrow in Fig. 14C).

**Expression of the fiber transgene in transgenic tobacco leaf and seed**

Fiber protein expression was detected in transgenic tobacco leaf as well as seed by western blot analysis. Tobacco leaf samples from two transgenic plant strains, named 35S-41 (containing 35S promoter and HEV fiber gene) and MeGA-9 (containing MeGA promoter and fiber gene) were selected for western blotting and ELISA using a turkey anti-HEV polyclonal serum absorbed by nontransgenic tobacco total protein. A mouse anti-HEV fiber monoclonal antibody was also used for sandwich ELISA. The kinetics of fiber protein accumulation for the inducible MeGA promoter was measured over time. The results (Fig. 15) revealed that the full-length fiber protein at 49 kDa was detected in both 35S-41 and MeGA-9 at almost the same expression level. For the transformant MeGA-9, there was no fiber protein expression before wound induction (0 hr). The 49 kDa band appeared as early as 8 hr incubation following wound induction (Lane 3 in Fig. 15). A second band at 35 kDa was observed in the 24 hr and 56 hr incubation samples with increasing amount. The 35 kDa band is probably a degradation product from the 49 kDa protein (see arrow, Fig. 15). Nontransgenic tobacco total protein was also tested as negative control. Although there was some cross-reaction between the nontransgenic tobacco protein and the turkey anti-HEV serum, there were no 49 kDa and 35 kDa bands in the negative control lane.

The expression level of the fiber protein in transgenic tobacco leaf was quantified by sandwich ELISA. HEV-A virus purified from turkey spleen was used as standard in the sandwich ELISA. The molecular weight of fiber protein is estimated at 1.45% of the whole virus capsid protein (Van den Hurk, 1992). In Fig. 16, the OD values of 35S-41 and MeGA-9 (24 hr incubation) are similar with that of the standard using 0.5 μg HEV-A. About 15 μg of total tobacco protein was used for each sample. By calculation: (0.5 μg X 1.45%) / 15 μg = 0.05%, the maximum level of fiber protein accumulation in the leaves of 35S-41 was 0.04% - 0.06% of the total soluble protein. A similar accumulation level was obtained for transformant MeGA-9 (24 hr incubation).

Total protein from transgenic tobacco (35S-41) seed was also analysised by Western blotting (Fig. 17). The results showed that fiber protein presents in seed (49 kDa) has 5 to 10 fold higher accumulation than in tobacco leaves and showed less degradation.

**Fiber protein purification from tobacco by immuno - affinity column**

In order to purify fiber protein from other tobacco proteins, absorbed turkey-anti-HEV serum was combined with CNBr – activated sepharose resin to make a immuno - affinity column. Fiber protein was eluted from the immuno - affinity column and was analyzed by western blotting using absorbed turkey anti-HEV polyclonal serum (see Fig. 18). Two bands at 35 kDa and 25 kDa were detected in eluted fractions (Lanes 3-4). The

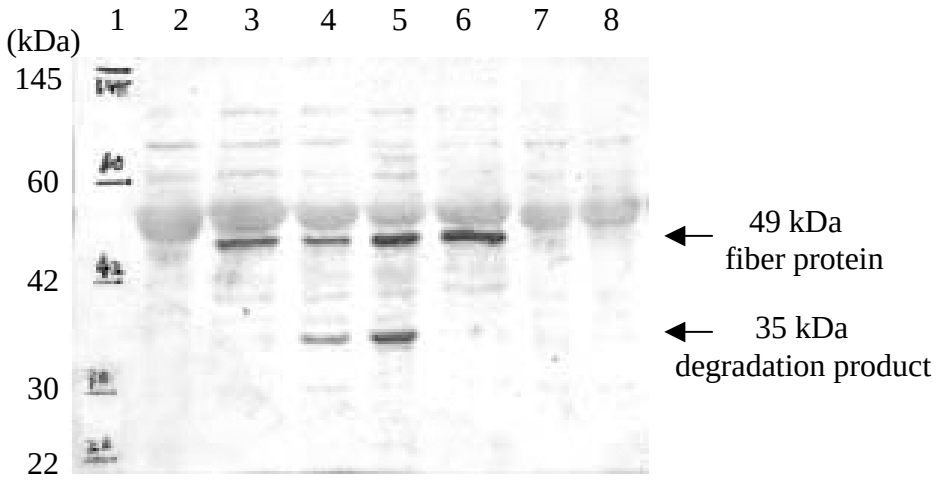



Fig. 15. Immunoblot analysis of fiber production in transgenic tobacco. Total protein (50 μg per lane) from transgenic and nontransgenic tobacco were gel separated, transferred onto membrane and reacted with absorbed turkey anti-HEV serum. Lane 1, molecular weight marker; lane 2, total protein from MeGA – 9 at 0 hr; Lane 3, total protein from MeGA – 9 at 8 hr; Lane 4, total protein from MeGA – 9 at 24 hr; Lane 5, total protein from MeGA – 9 at 48 hr; Lane 6, total protein from 35S – 41. Lane 7 – 8, total protein from nontransgenic tobacco as negative control.

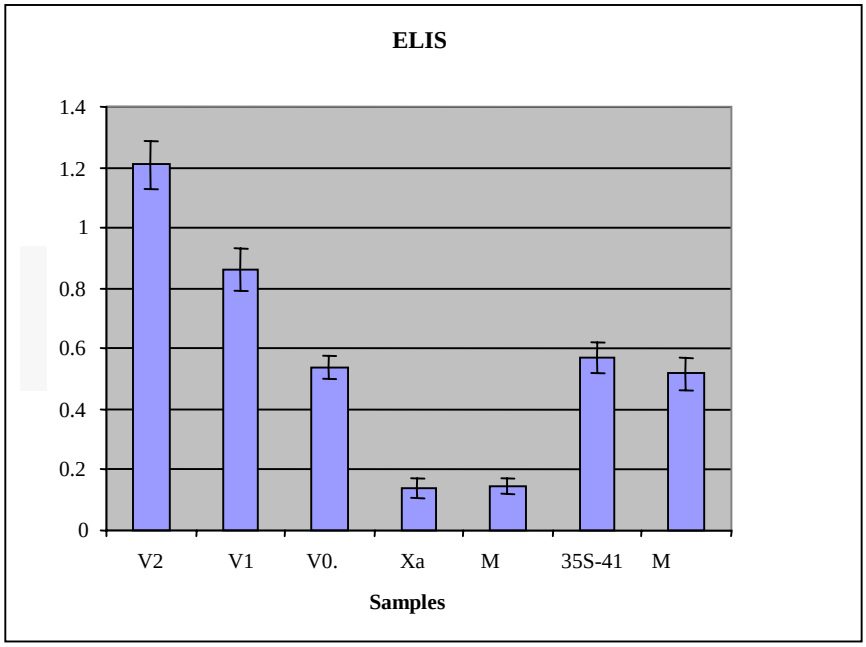



Fig. 16. Quantification of fiber protein from transgenic tobacco leaves by ELISA. The error bars represent triplicate wells for each sample. Positive controls are purified HEV-A. V2: 2 µg of HEV-A. V1: 1 µg of HEV-A. V0.5: 0.5 µg of HEV-A. Negative control is nontransgenic tobacco total protein extract (Xan). M 0hr: tobacco total protein (15 µg) from MeGA-9 with 0 hour of incubation. 35S-41: total protein (15 µg) from transformant 35S-41; M 24hr: total protein (15 µg) from tramsformant MeGA-9 at 24 hr of incubation.

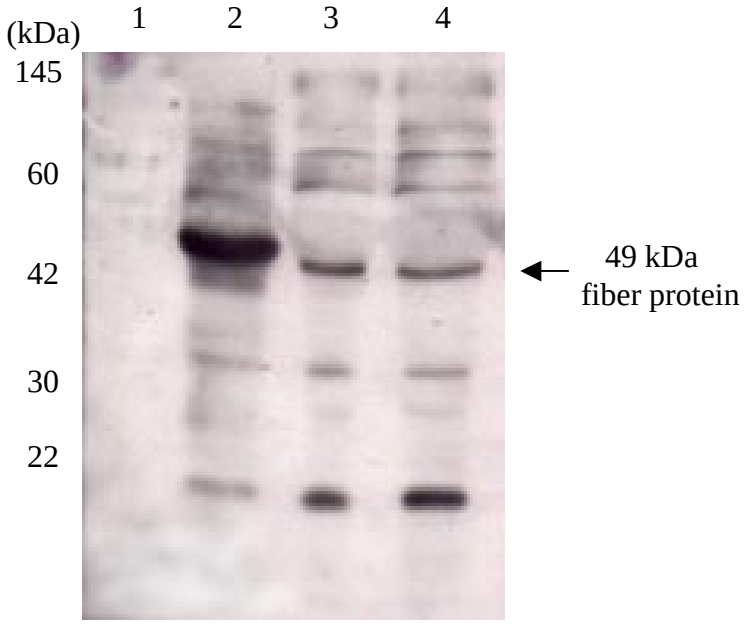



Fig. 17. Immunoblot analysis of fiber production in transgenic tobacco seeds. Total protein (50 μg per lane) from transgenic and nontransgenic tobacco seed as well as total protein from transgenic tobacco leaves were gel separated, transferred onto membrane and reacted with absorbed turkey anti-HEV serum. lane 1, total protein from nontransgenic tobacco leaves; Lane 2, total protein from 35S-41 seeds; Lane 3,4, total protein from 35S-41 leaves.

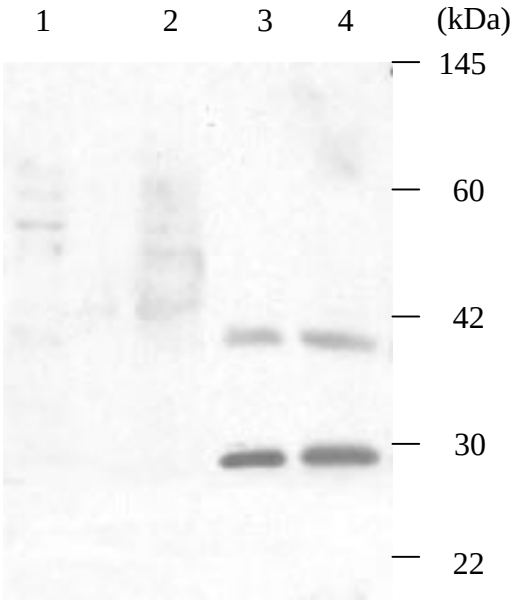



Fig. 18. Immunoblot analysis of plant – produced fiber protein purified by immuno affinity column. Eluates from the immuno affinity columns were gel separated and transferred onto membrane. The blot was then reacted with absorbed turkey anti-HEV serum. Lanes 1 - 2: eluate of nontransgenic tobacco; Lane 3: eluate of 35S – 41; Lane 4: eluate of MeGA – 9 at 8 hr after wounding.

native fiber protein is 29 kDa and based on our previous experimental results, the plant-produced recombinant fiber protein is about 49 kDa. The two protein bands (35 kDa and 25 kDa) are probably the degradation products of the 49 kDa fiber protein. There were no obvious bands in the negative control in which the nontransgenic tobacco total protein was used for the immuno-affinity column (Lanes 1-2).

**Rubisco crystallization**

Ribulose - 1,5-bisphosphate carboxylase / oxygenase (rubisco), which catalyses the central $CO_2$ - fixing reaction of photosynthesis in plants, is the most abundant protein in tobacco leaves (Bowman *et al.,* 1980; Kim *et al.,* 1997). The molecular weight of the native enzyme has been determined to be 520 kDa (Bowman *et al.,* 1980; Kim *et al.,* 1997). Under denaturing condition, it is about 55 kDa (Bowman *et al.,* 1980; Kim *et al.,* 1997). Rubisco is about 40% of the total soluble protein in plant protein extraction (Bowman *et al.,* 1980). In order to eliminate rubisco and enrich the fiber protein, the pH of protein extraction was changed to preferentially precipitate rubisco.

From the stained SDS-PAGE gel (see Fig. 19A), we can see that rubisco was precipitated by lowering the pH of the extraction buffer (pH 4.5). At the same time, almost 40% of other tobacco protein was also precipitated from the tobacco protein extraction. Western blot analysis (see Fig. 19B) showed rubisco was precipitated by lowering the pH. At pHs 4.0, 4.5, 5.0, there was no rubisco in the supernatant of protein extract. At pH 5.5, the rubisco cannot be crystallized and stays in solution. However, at the same time of rubisco crystallization, the fiber protein band also disappeared. It might be co-precipitated together with rubisco. This also suggested that the turkey-anti-HEV sera may show some non-specific binding to rubisco. At pH 5.5, although the rubisco is still detectable, the 49 kDa fiber protein band disappeared. There is a minor band at 35 kDa that is probably a degradation product of fiber protein; this could have occurred as the total protein extraction was placed at 4°C for overnight.

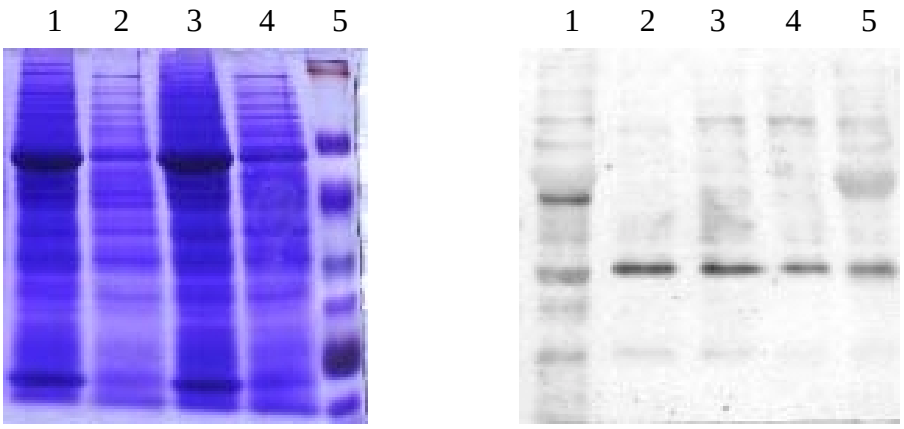



Fig. 19. Coomassie blue staining and immunoblot analysis of tobacco protein after the rubisco crystallization. Each sample was started with 50 μg of total tobacco protein. Same volume of each sample was loaded for each lane. (A). Effect of rubisco crystallization analyzed by SDS-PAGE. Proteins were gel separated and stained by coomassie blue. Lane 1: total protein before crystallization at pH 7.0; Lane 2: total protein after crystallization at pH 4.0; Lane 3: total protein before crystallization at pH 7.0; Lane 4: total protein after crystallization at pH 5.0; Lane 5: molecular weight marker. (B). Effect of rubisco crystallization analyzed by immunoblot. The membrane was reacted with absorbed turkey anti-HEV serum. Lane 1: total protein before crystallization at pH 7.0; Lane 2: total protein after crystallization at pH 4.0; Lane 3: total protein after crystallization at pH 4.5; Lane 4: total protein after crystallization at pH 5.0; Lane 5: total protein after crystallization at pH 5.5.

## Chapter IV
## Discussion

HEV fiber protein is a glycoprotein and is responsible for recognizing and attaching to the cell receptors on the surface of intestinal tract. The recombinant HEV fiber protein produced in *E. coli* failed to offer protection for turkeys against HEV-A challenge. HEV fiber that was expressed at high level in *E. coli* and used to inoculate turkeys did induce an antibody response. However, these antibodies did not recognize HEV-A virus (by western blot analysis) and failed to induce protection against HEV-A infection. Previous studies by Van den Hurk (1992) demonstrated that HEV hexon protein purified from viral particles served as an effective protective antigen in turkeys. They also have some experimental results to show that fiber is also a protective antigen for turkeys. There are several possibilities of why the *E. coli* -synthesized fiber protein did not offer protection. *E. coli* expression system is a prokaryotic expression system and lacks the machinery for protein glycosylation. Fiber protein is a glycoprotein. If critical antigen determinates are dependent on glycosylation, then fiber will need to be expressed in eukaryotic hosts. Fiber protein has a characteristic shape of a shaft terminated by a globular head. Human adenovirus serotype 3 (Ad3) fiber protein has the similar shape. Research showed that the Ad3 fiber protein expressed in *E. coli* with a 14-amino acid NH2-terminal fusion peptide could not form the correct shape. Under electronic microscopy, they saw a lot of heads and shafts of the fiber. However, those heads and shafts were not linked together (Corinne *et al.*, 1991). In contrast, Ad3 fiber protein expressed in *E. coli* without the tag had the head and shaft linked together. The results suggested that the fusion peptide at the $NH_2$-terminus prevented correct protein folding. The HEV fiber protein produced in *E. coli* was also fused with $NH_2$-terminal fusion peptides. The tag may interfere with the three dimensional structure of HEV fiber protein and thus not allow formation of protective epitopes.

Since the *E. coli*-produced fiber protein failed to induce protection in turkeys, we selected transgenic tobacco as a model to express the fiber protein in a eukaryotic system with potential for both bioproduction and oral delivery. Since the concept of using transgenic plants for vaccine production was first described by Mason *et al.* (1992), several research groups have described the expression of vaccine antigens using this methodology as discussed in the introduction. The demonstration that some of these antigens were immunogenic when orally administered encouraged the study of other antigens expressed in plants in order to develop edible vaccines. In the studies reported here, we showed that transgenic tobacco produced HEV fiber protein with a molecular weight of 49 kDa and two breakdown products (35 kDa, a 23 kDa). The levels of fiber protein accumulation in young leaves of the highest expressing plant lines, 35S-41 and MeGA-9, were 0.04 - 0.06% of total soluble protein. The molecular weight of native protein isolated from viral particles is 29 kDa. Based on the length of fiber gene coding region, its translation product should be around 49 kDa in molecular weight. Thus, the fiber present in viral particles has undergone post-translational processing although the mechanisms are currently unknown. Presence of 49 kDa product in plants suggest that the components for efficient processing to a 29 kDa form are not present in plants. Breakdown products of 49 kDa protein are seen in plants. N-terminal sequence of the

products is required to assess whether these products represent proteolylic processing similar to that occurring in avian cells.

The *E. coli*-produced fiber protein was also used to immunize a rabbit in order to obtain anti - fiber serum. When this rabbit serum was used to recognize plant - synthesized fiber protein, the 49 kDa fiber protein band shows significantly less cross-reactivity than observed with the turkey anti-HEV serum. The *E. coli*-produced fiber protein might have lost some important epitopes during the expression by *E. coli*. Thus the rabbit serum produced by immunizing a rabbit with *E. coli*-produced fiber protein cannot recognize well the plant - synthesized fiber protein.

Purified fiber protein from tobacco leaf was extracted by immuno-affinity column chromatography. Two bands at 35 kDa and 25 kDa were observed in eluted fractions. Rubisco was not present in the affinity column eluate. This experiment was repeated several times and the results were very consistent. During the procedure of purification, fiber protein was placed at 4°C overnight for binding to the column resin and at room temperature for several hours for elution. Since fiber protein is not very stable, it is likely that the degradation happened during that time.

Plant cells present differences in protein glycosylation with respect to animal cells. Glycosylation in plants may differ in the extent of glycosylation and processing (Faye *et al.,* 1993). The capsid glycoprotein of rabies virus (McGarvey *et al.,* 1995) expressed in tomato showed a molecular mass about 4 to 6 kDa less than the protein extracted from native virus (McGarvey *et al.,* 1995). However, the antigenic determinants seem to be preserved because the plant derived antigens induced neutralizing antibody in immunized mice. The presence or composition of glycans on the tobacco-synthesized fiber was not tested in this study.

This thesis demonstrated the feasibility of expressing HEV fiber protein in plants. Two promoters were used to drive the expression of fiber protein in transgenic tobacco. There was no significant difference in the amount of fiber protein accumulating in plant between the two promoters. Because the site of insertion of the transferred DNA into the chromosomal DNA is random, different levels of protein expression in independent transformants are expected. Expression levels were similar to that described with equivalent constructs expressing different virus capsid glycoproteins (Mason *et al.,* 1992; Gomez *et al.,* 1988; McGarvey *et al.,* 1995).

In order to make the idea of “edible vaccines” becomes a reality, several issues need to be addressed. Delivery of a consistent and efficacious vaccine “dose” with antigen levels sufficient to ensure protective immunity will be critical. Based on experimental results in this thesis, the highest expression of fiber protein in tobacco is 0.06% of total soluble proteins. In order to deliver a dose of 40 μg of plant-synthesized fiber protein, we need about 100 grams of fresh tobacco leaves for one immunization of one turkey. Crude tobacco proteins need to be extracted and concentrated to several microliters for injection. This procedure is time consuming and expensive. Thus, it is likely that higher level expression of HEV protein would be required for commercial applications. The use

of plant-derived leader sequences and signal peptides, and modification of the codon usage of this fiber protein could improve expression level in plants. Co-expression of penton base and fiber in plants may increase the chances of offering protection against the disease in turkeys. Self-assembly of penton base and fiber may yield antigen that is significantly more stable and, as a protein complex, may be more immunogenic and protective than the single fiber protein.

Fiber protein was also found in tobacco seeds at 5 to 10 times higher expression level and showed less breakdown products. This may reflect seeds natural ability to store and concentrate proteins and keep them stable. Seeds, incorporated into poultry pellets or used as a coating, could offer a convenient way to deliver oral vaccine. In order to have about 30 μg fiber protein to be delivered, we need about 10 mg of tobacco seeds for one dose.

However, research also shows that over-expression of certain foreign proteins may be detrimental to plant development. For example, transgenic potato expressing high levels of LT-B displayed slow shoot growth and poor tuber yield (Mason *et al.*, 1998). One possible solution is to use a tissue specific promoter. Potatoes expressing LT-B driven by a tuber-specific promoter showed normal shoot growth but still poor tuber development (Mason *et al.*, 1998). An alternative solution might be using an inducible promoter. Several inducible promoters being used in transgene plants including an ethylene inducible promoter (Clendennen and May, 1997) and the MeGA$^{TM}$ wound-inducible promoter (Cramer *et al.*, 1997). Many fruit ripening genes are ethylene inducible (Clendennen and May, 1997). When the fruit is ripening, the promoter is turned on and the plant starts to express the transgene. Since the plant is already mature, the foreign protein accumulation should not affect the plant development. In this thesis, the MeGA promoter was fused with fiber gene and transformed into tobacco. The expression level is about 0.06% of total tobacco proteins after 24 hours of induction. This expression level is almost the same as the protein yield accumulated in leaves from constructs driven constitutively by 35S promoter. The wound-inducible promoter is activated by mechanical stress such as cutting. The plant can be grown in the field and after harvest and transport to processing facilities, can be thinly sliced to trigger transgene protein production over the subsequent 48 hr. The ability to separate plant biomass production from recombinant protein production or to developmentally restrict production (e.g., in fruit or seeds) should enhance consistency of antigen yields and reduce environmental impacts and protein degradation.

Oral administration of plant based vaccines requires a higher dosage to induce the immune response (Mason *et al.* 1992). Both the amount of antigen per dose and administration schedule will be important to ensure triggering a protective response rather than oral tolerance. Researchers have tested the efficacy of using transgenic plants to direct oral tolerance rather than protective immunity in autoimmune diseases such as diabetes. Expression of glutamic acid decarboxylase (GAD), an autoantigen associated with diabetes, has been reported in tobacco and potato (Ma *et al.*, 1997). On a daily diet, nonobese diabetic (NOD) mice supplemented for four weeks with transgenic plant tissues expressing GAD were protected from onset of diabetes. Analogous results were obtained

by feeding NOD mice transgenic potato producing human insulin, a major insulin-dependent diabetes autoantigen (Arakawa *et al.*, 1998). NOD mice fed with transgenic potato tuber showed a significant reduction in insulitis (pancreatic islet inflammation), and suppression of diabetic symptoms. These experiments highlight the importance of dosing and appropriate administration schedules to ensure that an oral vaccine triggers the appropriate immune response. For protective immunity, increasing the amount of antigen for each dose and reducing the frequency of the dosage would be feasible. Co-expression of adjuvant or specific cytokines in the transgenic plant may amplify the immune response and ensure a protective response. Current research focuses on using LT-B or CT-B as adjuvants and/or carriers to enhance delivery and presentation of oral antigens (Arakawa *et al.*, 1997).

The choice of crop and its processing may also be critical in development of effective edible vaccines for both humans and livestock. Cooking and food processing may cause the protein to denature and lose its ability to elicit protective immune responses. For humans, this problem can be overcome by using plants such as tomato or banana that are eaten raw (Wong *et al.*, 1998). Corn and soybean are ideal for animals. These crops are all planted worldwide and could be available locally in many geographic areas. Recent research even showed that foreign proteins expressed in transgenic corn were stable enough to go through the standard corn seed processing conditions (Kusnadi *et al*., 1998). *E. coli* β–glucuronidase (GUS) and chicken egg-white avidin were expressed in corn seed at level of 5.7% and 0.7% of total protein, respectively. Both proteins were stable in seeds for at least 2 months at 10 °C and 3 weeks at 25 °C, which means that corn seed can be shipped below 25 °C to a different processing facility without loss of protein activity. The proteins were also stable for 1 week at 50 °C; 24 hours at 70 °C and 4 to 5 hours at 90 °C. Because the standard milling conditions include temperatures between 40 °C and 90 °C and contact times of less than 1 minute, this research demonstrated that conventional seed processing methods for feed corn may be suitable to process transgenic "vaccine" corn. Tobacco is not a component of animal feed but is often used in transgenic research due to ease of transformation. In research testing phosphorus utilization in poultry fed transgenic seed containing phytase (an enzyme enhancing phosphorus availability), transgenic tobacco seeds were milled and added to the diet of broilers over a 4-week period. Transgenic seed-based delivery of phytase was as effective as direct enzyme or inorganic phosphate supplementation (Pen *et al.*, 1993). Of significance for vaccine development, tobacco seeds were readily consumed and had no adverse effects on animal health. Thus, transgenic tobacco seed may provide a stable and convenient delivery system that is directly applicable in animal feed for the short-term administration needed for a vaccine. However, the first transgenic plant-based edible vaccines available commercially will likely be transgenic corns targeting an important livestock disease. In the future, vaccination of animals could be as simple as supplementing their feed with the appropriate transgenic plant material.

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