

DEVELOPMENT OF PLANT-BASED VACCINE FOR NEWCASTLE DISEASE IN POULTRY

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Newcastle disease is a highly contagious zoonotic bird disease affecting many domestic and wild avian species. Its effects are most notable in domestic poultry due to their high susceptibility and the potential of becoming endemic in many countries. The causal agent, Newcastle disease virus (NDV), is a negative-sense single-stranded RNA virus. Transmission occurs by exposure to faecal and other excretions from infected birds, and through contact with contaminated feed, water, equipment and clothing (Alexander, D.J. 1991). Signs of infection with NDV vary greatly depending on factors such as the strain of virus and the health, age and species of the host.

The development and introduction of new vaccines for developing countries faces many challenges. The vaccines must address the need for lower costs, oral-activity, heat stability, and mucosal effectiveness. An exciting vision is to use transgenic plants as very low cost, highly-efficient production systems, especially suitable for initial development and production in developing countries, of orally-active antigens that will be prepared for oral administration, controlled to meet appropriate regulatory requirements, and supplied as safe and effective vaccines (Kaufmann, 2004).

Studies have already shown that genetically engineered plants can produce vaccine which can cause an immunological response in mice that have eaten these plants. Plant could act as natural bioreactor in producing large amounts of proteins without the huge investments in setting up the facilities for processing and harvesting. Transgenic plants that express foreign proteins with industrial or pharmaceutical value represent an economical alternative to fermentation-based production systems. In addition, contamination with animal viruses can be eliminated, since cultured cells will not be used in the production process. Many of the quality control tests that require animals also could be eliminated. Specific vaccines have been produced in plants as a result of the transient or stable expression of foreign genes. It has recently been shown that genes encoding antigens of bacterial and viral pathogens can be expressed in plants in a form in which they retain native immunogenic properties. Thus, similar efforts have been focused towards developing a suitable strategy to develop a plant-based vaccine for NDV. In order to achieve this, we have set the following objectives:

1. To construct a plant vector system carrying viral fusion (F) gene of NDV.
2. To transform *Nicotiana tabaccum* plant with recombinant F gene of NDV.
3. To analyze the expression of the transgenes of the plant-based NDV vaccine.

The development of a plant-based vaccine against NDV would benefit the poultry industry and would improve animal health.

MATERIALS AND METHODS

The overall experimental strategy for the development of recombinant plant-based NDV vaccine involves the preparation of cDNA from NDV strain V4, followed by PCR amplification of FI, F1P, F2 and F2P genes using the following primer pairs: a) F1 [For: 5'-CCATGGTTATAGGCGCCATTATCGGTGG-3'; Rev: 5'-AGATCTGTCACTATTA CTTGAGAATCTTGTATTGAGATATTC-3']; b) F1P [For: 5'CCATGGCCAATCAAA ATGCTGCCAACATACTCC-3'; Rev: 5'-AGATCTGTCACTATTACTTGAGAATCTT GTATTGAGATATTC-3']; c) F2 [For: 5'-CCATGGGGCTCCAGGTCTTCTACC AG-3'; Rev: 5'-AGATCTCCCTGTTTCCCTCCTCCGG-3']; d) F2P [For: 5'-CCATG GCCC TTGATGGCAGGC-3'; Rev: 5'-AGATCTCCCTGTTTCCCTCCTCCGG-3']. Primers introduced start codons at 5' end of the genes and *NcoI* sites at the 5' end of the F genes and *BglII* sites at the 3' ends of all the different fragments of the F gene. The amplified products were cloned into pGEMT Easy (Promega) and sequenced. The sequences were evaluated to ensure that no changes of nucleotides occurred in the amplified product. The different fragments the F gene were then subcloned into pET28a for expression studies. Following that the construction of plant vector system were carried out using ImpactVector 1.1 (without HDEL) and (1.3 with HDEL). The expression of the F1, F1P, F2 and F2P in *E. coli* were carried using Rosetta cell (*E. coli*), whereas the expression in plant were performed in *Nicotiana benthamiana* using MagnICON system (Icon Genetics, Germany). Following that the F gene which showed the presence of soluble recombinant F protein of NDV was selected and used for stable plant transformation study. *Nicotiana tabacum* was transformed with *Agrobacterium tumefaciens* strain LBA4404.

Plasmid constructs for leaf disc transformation

The ImpactVectors carrying the F2 and F2P genes of NDV were obtained by double digestion of the 300 ng of the plasmid with *Ascl* and *PacI* and both the fragments were subcloned separately into the same enzyme site in the binary vector pBinPlus (ImpactVector, Wageningen, Netherland), that contain kanamycin gene as selectable marker and the right and left borders necessary for T-DNA transmission to obtain plasmid pBinPlus-F2 and pBinPlus-F2P. The resultant plasmids pBinPlus-F2 and pBinPlus-F2P were introduced in *Agrobacterium tumefaciens* strain LBA4404 pAL4404 using the electroporation method described by Wen-Jun et al (1989). Electroporation of the *Agrobacterium rhizogenes* strain A4 was performed using Eppendorf electroporator (model 2510) at 1800kV

Transient plant transformation

The magnification procedure was used for rapid production of recombinant antigen (Gleba et al., 2005, Gleba et al. 2007, Golovkin et al., 2007). *Agrobacterium tumefaciens* strain GV3101 cultures carrying the expression cassette were mixed with culture carrying pre-manufactured helper plasmids (*pICH14011* and *pICH17620*) (Icon Genetics) and applied to two months old *Nicotina benthamiana* plants. Plant tissues were harvested after 9 days, processed according the method described by Brodzik et al. (2006) and Golovkin et al (2007), and analysed by Western blot.

Plant Transformation

Leaf disc of *Nicotiana tabaccum* were infected with *Agrobacterium tumefaciens* strain LBA4404 pAL4404 (OD₆₀₀ 0.4) for 10 min. Following that, the infected leaf discs were co-cultured for over a period of 48 hours on MS medium (Murashige and Skoog, 1962) containing 100µm acetosyringone. The explants were then subcultured onto regeneration medium (MS supplemented with 2 mg/l BAP, 0.1mg/l NAA, 100mg/l kanamycin and 300 mg/l carbenicillin, pH 5.8) and transferred to fresh medium every 15 days until distinct shoots appeared. Finally, shoots were grown in micropropagation medium (MS supplemented with 2 mg/l BAP, 100mg/l kanamycin and 300 mg/l carbenicillin). Following that, the individual plantlets were separated and subcultured onto hormone free MS media containing 100mg/l kanamycin and 300 mg/l carbenicillin. All plants were maintained by periodic micropropagation.

Molecular characterization of the transgenic events

All DNA extractions were carried out according to Dellaporta et al (1983). PCR was performed on total DNA extracted from kanamycin-resistant plants to confirm the presence of the F2 genes using forward primer (5'-CCATGGGCTCCAGGTCTTCTACCAG-3') and reverse primer (5'-AGATCTCCCTGTTTCCCTCCTCCGG-3') and F2P genes using forward primer (5'-CCATGGCCCTTGATGGCAGGC-3') and reverse primer (5'-AGATCTCCCTGTTTCCCTCCTCCGG-3')

RESULTS

To obtain a candidate plant-based recombinant vaccine against Newcastle disease virus in poultry, we have looked into the two main components of the viral genes, which is the F1 and F2 regions of the NDV strain V4. From each of the two genes, two different fragments were amplified. The F1 (consist of entire F1 region), FIP (consist of the F1 region minus the hydrophobic transmembrane anchor), F2 (consist of the entire F2 region with neutralizing epitope) and F2P (consist of the entire F2 region with neutralizing epitope minus the N-terminal signal peptide) genes of NDV are approximately 1023bp, 939bp, 350bp and 260bp, respectively and has *NcoI* restriction site on the 5'-end and *BglII* restriction site on the 3'-end. These Fusion gene fragments were successfully cloned into *pBIV1.1Tag* vector as a translational fusion with the cytoplasmic signal peptide; c-myc and His₆ tag epitopes, and *pBIV1.3Tag* vector as a translational fusion with the ER signal peptide; c-myc and His₆ tag epitopes and the ER retention signal

HDEL (Plant Research International, Wageningen, NL) were attached to the NDV F gene C-terminus. Following that, these fragments were individually subcloned into pET28a at the *Nco/SacI* sites and transformed into *E.coli* DH5 α for multiplication and *E. coli* Rosetta cell for expression studies. Gel electrophoresis of the amplified product of the above genes showed clearly the presence of the different constructs carrying fragments of F genes. Expression studies performed using the rosetta cells showed that expression of the different Fusion protein of NDV were detectable even as low a 5 μ g of soluble protein.

Transient transformation studies were also performed on *Nicotiana benthamiana*. The result showed that after 9 days, the expression of different fusion genes were detectable using the immunological detection system. However, most of the proteins were insoluble. Only a very small amount of soluble protein was detected in all the different fragments of F1 and F2 genes. Observation of the F2P genes, however, interestingly showed the presence of 2 protein bands at 17 kD and > 80 kD. Based on the actual size of the F2P gene, the predicted size of F2P is approximately 10 kD. Following this observation, only F2P was selected for the stable transformation. The antigens (F2P protein) expression cassette containing promoter and termination signal was placed in the *pBINPlus* binary vector, yielding the constructs *pCF260* and *pRF260*. Both, *pCF260* and *pRF260* were transformed into *Nicotiana tabaccum*. Several stably transformed transgenic tobacco lines were selected based on kanamycin resistance and PCR analysis. The transformation efficiencies of *pCF260* and *pRF260* were 30% and 50%, respectively. On the average, 70% of the transgenic tissue cultured plants were successfully acclimatized and transferred to the glasshouse. The expression studies on the F2 fusion protein in the stably transformed tobacco plants, however, are currently in progress. Following that, the immunogenicity of the F2P protein expressed will also be determined.

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