Original article

CLINICAL AND EXPERIMENTAL VACCINE RESEARCH

Transient expression of fusion and hemagglutinin-neuraminidase epitopes of Newcastle disease virus in maize as a potent candidate vaccine

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Purpose: Newcastle disease (ND) represents a major viral disease across the world which imposes high costs to poultry producers for vaccination. Hemagglutinin-neuraminidase (HN) and fusion (F) proteins are the major immunogenic epitopes of Newcastle disease virus and hence, have been the main targets for development of anti-ND vaccines. This paper reports transient expression of a synthetic gene composing of four tandem repeats of HN and three tandem repeats of F epitopes in maize leaves as initial step toward production of recombinant vaccine against ND.
Materials and Methods: The synthetic gene was cloned in pBI121 plasmid to yield an expression vector. The vector was sophisticated by the addition of AUG codon, polyhistidine-tag, tobacco mosaic virus omega sequence, stop codon, and restriction sites. Leaf transformation was conducted by the agroinfiltration method. Molecular detection assays including polymerase chain reaction, reverse transcription-polymerase chain reaction and enzyme-linked immunosorbent assay (ELISA) were carried out to evaluate transgene expression in infiltrated leaves of the corn plant.
Results: The result obtained in this research revealed that the transgene was transcribed and translated in maize leaves only 48 hours after infiltration. In the second phase of the experiment, the expressed protein was injected into rabbits. The result of the ELISA assay indicated induction of immune response in the rabbits after injection with the heterologous protein.
Conclusion: These results confirm the feasibility of agroinfiltration for transient gene expression of viral epitopes in monocot plants which naturally resist stable transformation by *Agrobacterium tumefaciens*. Practical implications of this finding are discussed in detail and some recommendations for future studies are proposed.

Keywords: Newcastle disease, Recombinant vaccine, Transient gene expression, Agroinfiltration, Maize

Introduction

Despite numerous advantages of green plants as natural factories for the development of recombinant vaccines, the high cost and long time required for the generation of transgenic plants have put significant hurdles in the way of commercialization of plant-based recombinant vaccines [1]. Transient gene expression has been used by many researchers to address the aforementioned problem. Applying transient gene

expression assay coupled with reliable detection methods significantly reduces the time required for the evaluation of transgene functionality in plant hosts [2]. Indeed, time- and cost-saving are two major advantages of the transient gene expression approach which make it an ideal choice for the initial evaluation of a candidate gene nominated for production of recombinant vaccine [3]. Furthermore, transgene delivered by transient gene expression method quickly stimulates transcription and translation machineries of the host cell which results in high accumulation of the desired protein [4]. Transient gene expression can be realized by various approaches including application of viral vectors, hairy root culture, agroinfiltration, as well as others [5]. Agroinfiltration represents the simplest method for transient gene expression. This method includes simply an injection of *Agrobacterium tumefaciens* suspension into plant leaves. Owing to its simplicity and reliability, agroinfiltration has been used by many authors for transient expression of the genes nominated for the production of recombinant vaccines [6].

There have been many attempts for the development of recombinant vaccines against the poultry Newcastle disease (ND). Hemagglutinin-neuraminidase (HN) and fusion (F) are the major immunogenic epitopes of Newcastle disease virus (NDV) and hence, have been the main targets for the development of anti-ND vaccines [7]. In this direction, many plant species have been tested by researchers for their utility as the platform for the production of anti-ND vaccines [8]; but the range of plant host for this purpose is often limited to dicotyledon species. In contrast, monocots are rarely used for the production of recombinant proteins since they are not a natural host of *A. tumefaciens* which is the main tool for genetic engineering. Corn is a monocot plant that serves both as a fundamental part of poultry diets and as an appropriate platform for the production of recombinant vaccines [9]. As mentioned by Karaman [10], maize is a suitable host for the production of various recombinant vaccines and vaccine components. Since agroinfiltration can be used for transgene expression in both monocots and dicots, we investigated the utility of agroinfiltration for transient expression of HN and F proteins in maize (*Zea mays*) leaves. Since maize is a food source for poultry, developing an anti-NDV vaccine in this crop may be an interesting topic for research. For this purpose, a synthetic gene comprising of tandem repeats of F and HN epitope was constructed and used for transient expression in maize leaves.

Most of the recombinant vaccines' success depends on their ability to induce immune responses in animal hosts. As is the case for traditional vaccines, targeting antibody-mediated immunity in the hosts is a prerequisite for introducing a new recombinant vaccine. For this purpose, the final phase of many recombinant vaccine development projects is to verify the ability of recombinant proteins in the induction of specific antibodies in animal models [3,7,11]. Based on this, at the final step of this experiment, the recombinant F protein was extracted and subcutaneously injected to rabbit as a common animal model in laboratory experiments. The ultimate goal of this research was to evaluate the feasibility of a transient gene expression approach for the development of plant-based recombinant vaccines against ND.

Materials and Methods

Construction of expression vector

It was tried to design a sophisticated gene construct with high expression potential in plant tissues (Fig. 1). For this purpose, coding sequence including four tandem repeats of HN epitope followed by three tandem repeats of F epitope was used as the core body of gene construct which was further elaborated with the addition of AUG codon, polyhistidine-tag, tobacco mosaic virus omega sequence as ribosome binding site and *BamHI* restriction site upstream of the coding sequence. Stop codon and *SacI* restriction site were also attached to 3′ end of the gene. The gene construct was cloned in the binary vector pBI121 containing CaMV 35S promoter and NOS terminator and transferred into AGL0 strain of *A. tumefaciens*.

Agroinfiltration

For agroinfiltration, overnight culture of *A. tumefaciens* strain AGL0 harboring pBI121-NDV epitopes vector was centrifuged and the bacterial pellet was collected after discarding the supernatant. Infiltration medium was prepared by mixing 10 mM $MgCl_2$, 10 mM MES pH 5.6, and 150 μM acetosyringone. The bacterial pellet was resuspended in an infiltration medium. The infiltration medium containing *A. tumefaciens* cells was left at room temperature for 2 hours. Then the infiltration medium was injected into maize leaves with a needle-free syringe (Fig. 2). Maize seedlings were placed in a growth chamber for 2 days and then analyzed.

Molecular detection

A polymerase chain reaction (PCR) assay was conducted to

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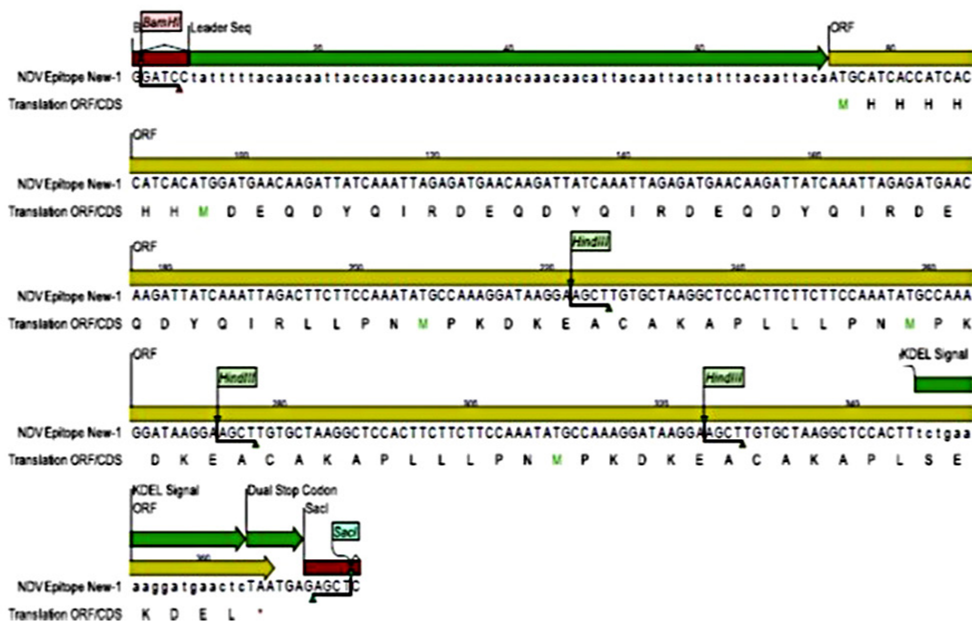


Fig. 1. Schematic presentation of the gene construct.

Fig. 2. Agroinfiltration of maize leaves using needle-free syringe.

investigate the presence of the transgene in infiltrated leaves. DNA from infiltrated and wild-type leaves was extracted and used for PCR analysis using specific primers of 5´TCATTGC-GATAAAGGAAAGGC 3´ and 5´ AATGTATAATTGCGGGACTC 3´. PCR was carried out by 35 cycles of 94°C for 60 seconds, 54°C for 60 seconds, and 72°C for 60 seconds, followed by a final extension step of 72°C for 10 minutes.

Expression of the synthetic gene was evaluated at the transcription level by reverse transcription-polymerase chain reaction (RT-PCR) assay. Total RNA was extracted from 500 mg of leaf tissue according to the general process of RNA extraction. Complementary DNA (cDNA) was synthesized via reverse transcription using an oligo $(dT)_{20}$ primer. The resulting cDNA mixtures were used as templates for RT-PCR. Forward and reverse primers for RT-PCR were 5´ ACTATTTACAATTA-CAATGCATCAC 3´ and 5´ GAGTTCATCCTTTTCAGA-AAGTG 3´, respectively.

Enzyme-linked immunosorbent assay (ELISA) plate was coated with total soluble proteins from the wild type and the transformed leaves at 37°C for 1 hour; followed by incubation with 1% bovine serum albumin in phosphate-buffered saline (PBS) for 2 hours at 37°C to prevent non-specific binding. The well was washed by PBST/PBS and incubated with horseradish peroxidase-conjugated with anti his tag (1:1,000). Wells were developed with TMB substrate; the color reaction was stopped by 2N H_2SO_4 and read at 450 nm of wavelength.

Induction of immune response in rabbit

Four rabbits were used from which blood samples were taken. Each serum was used with three replications. Rabbits were immunized at days 0, 28, and 40 with 150 µL of crude protein of wild type and transgenic plants emulsified in chitosan adjuvant. Animals were then bled and the presence of anti-NDV specific antibodies in sera was determined by ELISA, in which the plates were coated with purified NDV (LaSota Newcastle vaccine). In summary, ELISA plate wells were coated with the LaSota Newcastle vaccine. After incubation and subsequent washing, PBS-Tween-20 was added and the plate was incubated and washed again. Then immunized

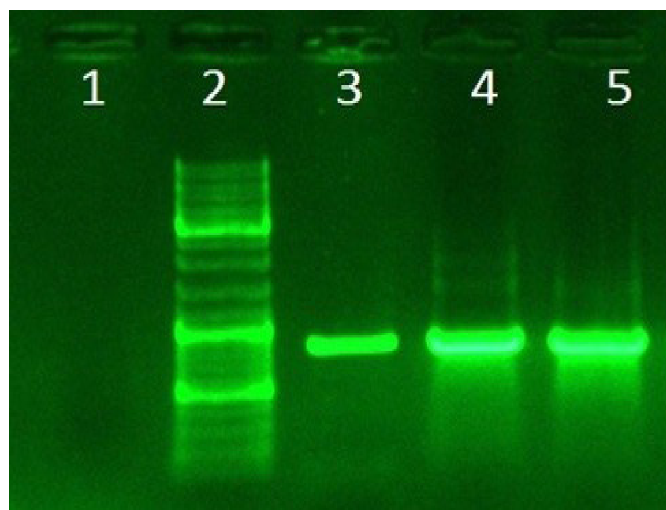


Fig. 3. Polymerase chain reaction detection of recombinant gene in transformed leaves. 1: wild type; 2: ladder, 3–4: transformed leaves; 5: plasmid pBI121-NDV.

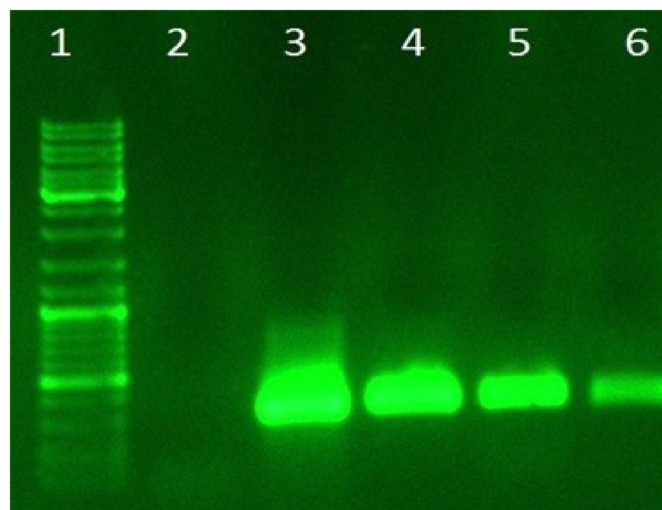
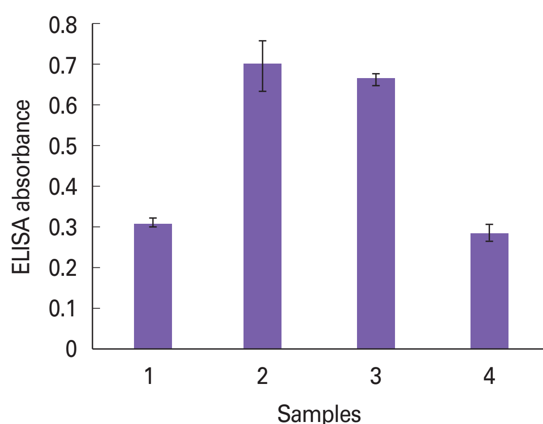


Fig. 4. Results of reverse transcription-polymerase chain reaction assay. 1: size marker; 2: wild type; 3: positive control; 4–6: infiltrated leaves.



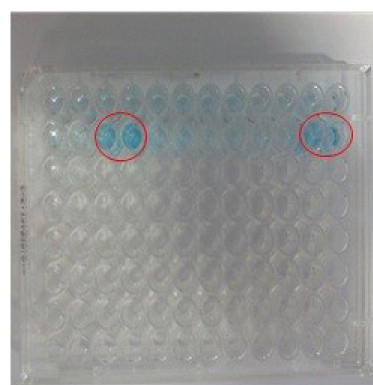


Fig. 5. (A, B) Detection of recombinant protein in infiltrated leaves using enzyme-linked immunosorbent assay (ELISA). 1: wild type; 2 and 3: protein samples of infiltrated leaves; 4: bovine serum albumin. The wells surrounded by red circles indicates 2 and 3 (protein samples of infiltrated leaves).

rabbits' sera were added as follows: rabbits immunized with commercial vaccine V4 (as positive control); rabbits immunized with PBS/chitosan (as test control); serum of rabbit immunized by the injection of infiltrated leaves; serum of rabbit immunized by the injection of non-infiltrated leaves (as negative control). Then anti-rabbit immunoglobulin G conjugated with alkaline phosphatase was added. After incubation and washing, optical density was measured at 405 nm by an ELISA reader.

Results

The result of PCR analysis aiming at verification of transgene presence in infiltrated leaves is depicted in Fig. 3. As seen in the agarose gel, an 810 bp band was detected in infiltrated leaves; confirming the presence of the foreign construct. No band was observed in the DNA of wild-type leaves. RT-PCR was conducted to evaluate the expression of F and HN epitopes in infiltrated leaves. The result of the RT-PCR assay is shown in Fig. 4. The results indicate that F and HN epitopes were expressed in maize leaves into mRNA in infiltrated leaves; indicating successful transcription of the foreign gene. Expression of the transgene at translation level was investigated by ELISA. The results of this assay are depicted in Fig. 5. Sharp signals were observed in protein samples obtained from infiltrated leaves and from positive control (5% level of significance); however, no signal was seen in the sample obtained from wild-type leaves. This assay shows specific anti-

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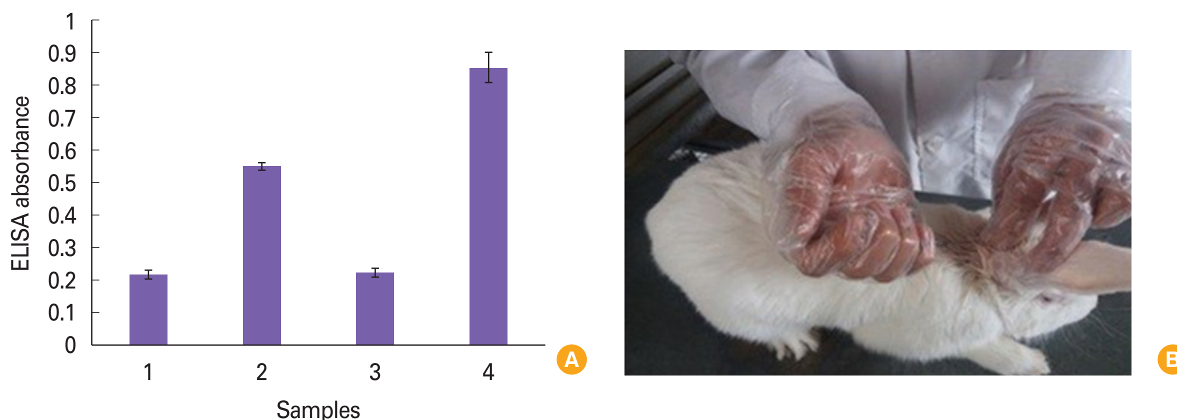

Fig. 6. (A, B) Immunogenicity study in rabbit sera using enzyme-linked immunosorbent assay (ELISA). 1: injected rabbit sera with phosphate-buffered saline with chitosan; 2: serum of rabbit immunized by injection of infiltrated leaves; 3: serum of rabbit immunized by injection of non-infiltrated leaves; 4: injected rabbit sera with V4 vaccine as positive control.

gen/antibody reactions for protein samples from infiltrated leaves and hence confirms that the transgene was translated into protein in transformed tissues. Moreover, quantification of transgene revealed production of the interested peptide as 0.016 mg/g fresh leaf.

ELISA assay was used for evaluation of anti-NDV antibodies in the rabbits intraperitoneally injected with crude protein of infiltrated leaves and that of wild-type plants (control group). The results indicated sharp signals in the sera of rabbits treated with transformed plant extracts (5% level of significance); while no detectable signal was observed in the sera obtained from the animals injected with extract of wild-type plants (Fig. 6).

Discussion

The present study was conducted to evaluate agroinfiltration as an efficient approach for the expression of viral epitopes in plant tissues as an initial step for the development of recombinant vaccines. Various types of transient gene expression have been used by many research teams as a pilot attempt for the production of recombinant subunit vaccines in green plants. For example, Wigdorovitz et al. [12] used a viral vector for the expression of VP1 antigen, a structural protein of the foot and mouth disease virus (FMDV). An oncoprotein of the human papillomavirus (HPV) was also expressed in *Nicotiana benthamiana* plants using viral vectors [13]. Kumar al. [14] used hairy root culture for the expression of hepatitis B surface antigen.

The results obtained in the present study demonstrated that agroinfiltration can be an efficient technique for the development of subunit vaccines. The results of RT-PCR and ELISA assays confirmed that the foreign gene was successfully transcribed and translated into protein in infiltrated leaves of maize; implying the applicability of agroinfiltration for the development of subunit vaccines against ND. These findings accord with those reported by other authors who applied agroinfiltration for expressing target antigens in plant tissues. For example, Habibi-Pirkoohi et al. [15] expressed VP1 epitopes of FMDV in tobacco (*N. tabacum*) using agroinfiltration and observed that the antigen was expressed in the infiltrated leaves. A high level of VP1 expression was also achieved by agroinfiltration in infiltrated leaves of *Spinacia oleracea* [16]. Similarly, Wroblewski et al. [17] successfully expressed foreign genes in lettuce, tomato, and *Arabidopsis* by means of agroinfiltration. In a comprehensive study, Chen et al. [6] described the feasibility of agroinfiltration for transient expression of numerous proteins with pharmaceutical value. Overall, the results obtained in the present experiment and those previously reported by other authors demonstrate the efficiency of agroinfiltration as a reliable technique for the expression of antigens nominated for the development of recombinant vaccines. Particularly, induction of immune response in animal models put further support for the feasibility of transient gene expression system for evaluation of immunogenicity of candidate recombinant vaccines. Induction of immune response in the animal host is the final step of laboratory effort in every project of recombinant vaccine development. An antigen expressed in the transgenic plants can be considered as a potential recombinant vaccine only if its

ability to induce immune response is approved through animal model trials [18]. The literature in the field of plant-based recombinant vaccines implies that immunological assay is a crucial step that paves the road for the probable practical use of recombinant proteins expressed as vaccine candidates [3]. Mammalian animal models are widely used by research groups to evaluate the immune-inducing potential of recombinant vaccines including anti-NDV vaccines. For example, Berinstein et al. [19] and Lai et al. [20] verified the immune-inducing potential of recombinant anti-NDV vaccines in mammalian animal models.

As stated elsewhere, recombinant vaccines are realized by the capacity of defined antigens to induce immunity against pathogens [11], the property which is verified through conducting immunological assays in animal models.

In conclusion, our experiment can be regarded as an attempt toward the development of new recombinant vaccines against NDV. The novelty of this study lies in the application of tandem repeats of HN and F epitopes which are the most immunogenic and hence the most common epitopes produced by heterologous expression systems for the development of anti-NDV vaccines. For example, Gómez et al. [21] expressed HN epitope in *N. benthamiana* by agroinfiltration and observed that the antigen was successfully expressed in the infiltrated leaves. Lai et al. [7] expressed HN glycoprotein in transgenic tobacco cell cultures and reported that the transgene was expressed at a high level in the cell suspension. Berinstein et al. [19] showed that NDV epitopes expressed in potato plants have the ability to induce immune responses in mice. Shahriari et al. [22] expressed HN and F epitopes of NDV in hairy roots of tobacco and observed that the epitopes are expressed at a high level in hairy root cultures.

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