

Expression of H3N2 nucleoprotein in maize seeds and immunogenicity in mice

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Abstract

Key message Oral administration of maize-expressed H3N2 nucleoprotein induced antibody responses in mice showing the immunogenicity of plant-derived antigen and its potential to be utilized as a universal flu vaccine.

Abstract Influenza A viruses cause influenza epidemics that are devastating to humans and livestock. The vaccine for influenza needs to be reformulated every year to match the circulating strains due to virus mutation. Influenza virus nucleoprotein (NP) is a multifunctional RNA-binding protein that is highly conserved among strains, making it a potential candidate for a universal vaccine. In this study, the NP gene of H3N2 swine origin influenza virus was expressed in maize endosperm. Twelve transgenic maize lines were generated and analyzed for recombinant NP

(rNP) expression. Transcript analysis showed the main accumulation of rNP in seed. Protein level of rNP in T1 transgenic maize seeds ranged from 8.0 to 35 µg of NP/g of corn seed. The level increased up to 70 µg of NP/g in T3 seeds. A mouse study was performed to test the immunogenicity of one line of maize-derived rNP (MNP). Mice were immunized with MNP in a prime-boost design. Oral gavage administration showed that a humoral immune response was elicited in the mice treated with MNP indicating the immunogenicity of MNP. NP-specific antibody responses in the MNP group showed comparable antibody titer with the groups receiving positive controls such as Vero cell-derived NP (VNP) or alphavirus replicon particle-derived NP (ANP). Cytokine analysis showed antigen-specific stimulation of IL-4 cytokine elicited in splenocytes from mice treated with MNP further confirming a TH2 humoral immune response induced by MNP administration.

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Introduction

Influenza A viruses are the causal agent of influenza epidemics that results in millions of dollars of economic loss and provokes serious health impacts due to morbidity to mortality. These viruses are found in many host organisms including birds (ducks and chickens), pigs, dogs and humans (Medina and García-Sastre 2011). Influenza A virus is a negative-sense RNA virus consisting of eight single-stranded RNA fragments that encode for 11 or 12 viral proteins (Portela and Digard 2002; Hutchinson et al. 2010; Medina and García-Sastre 2011). Influenza A viruses are

classified into subtypes based on the amino acid sequence of the hemagglutinin (HA) and neuraminidase (NA) glycoproteins. Currently, there are 16 HA subtypes and 9 NA subtypes of influenza viruses circulating in birds, while two strains H1N1 and H3N2 commonly circulate in humans (Medina and García-Sastre 2011).

The current practice of influenza vaccination is based on the surface antigens HA (Crawford et al. 1999; Steel et al. 2010; Kodihalli et al. 1999) and NA (Chen et al. 2000; Brett and Johansson 2005), which are the major proteins involved in the receptor binding to the host cells and release of progeny virions from host cells, respectively (Medina and García-Sastre 2011; Chen et al. 2009). However, these glycoproteins are prone to mutation due to antigenic shift and antigenic drift (Luo et al. 2012; Medina and García-Sastre 2011). These mechanisms have allowed the virus to develop into a new strain that may or may not result in cross-species transmission (Kuiken et al. 2006). For example, the swine origin 2009 H1N1 virus caused pandemic in humans (Medina and García-Sastre 2011; Garten et al. 2009). Consequently, the human influenza vaccine has to be reformulated each year to match the current circulating strain, because previous vaccines may not be effective for the new strain.

Many studies have focused on the development of a universal vaccine based on conserved proteins such as the extracellular matrix protein (M2e), HA and nucleoprotein (NP). Conserved proteins are less likely to undergo mutation and thus, the vaccine does not need to be changed each year (Du et al. 2010). The purpose of a universal vaccine is to provide broad protection against heterosubtypic strains of influenza virus. This is important due to the fact that the development of strain-specific vaccines can take months. Therefore, the universal vaccine can prevent or reduce the effects of an epidemic before the strain-specific vaccine becomes available (Zheng et al. 2014).

The sequence of the NP gene is 90 % conserved among influenza virus strains and types (Portela and Digard 2002; Epstein et al. 2005). This protein plays a role in virus genome transcription and replication (Turrell et al. 2013). Previous studies showed that NP protein is the major target for cross-reactive immune responses and a potential candidate for a universal vaccine (Yewdell et al. 1985; Townsend et al. 1985). Vaccination with NP protein (Wraith et al. 1987) or NP DNA (Luo et al. 2012; Epstein et al. 2002) in mice led to generation of NP-specific cytotoxic T cells and virus clearance. Recombinant NP (rNP) has been expressed using different systems such as animal cells (Townsend et al. 1984), bacteria (*Escherichia coli*, *Salmonella typhimurium*) (Huang et al. 2012; Ashraf et al. 2011) and virus vectors (Crawford et al. 1999; Epstein et al. 2005). However, there are limited studies related to immunogenicity of NP protein produced in plants.

Plant molecular farming is considered as a feasible and relatively inexpensive system for large-scale production of pharmaceuticals (Ma et al. 2003). Transgenic plants are favorable over animal systems due to properties of scalability, speed, quality, and freedom from animal pathogens (Perea Arango et al. 2008; Rybicki 2010; Rosales-Mendoza and Salazar-Gonzalez 2014). The attempts and success of pharmaceutical protein production such as antibodies, enzymes, antigens, subunit vaccines and epitope peptides in plants have been reviewed elsewhere (Daniell et al. 2001; Ma et al. 2003; Daniell et al. 2009; Stoger et al. 2014). Several plant-made pharmaceutical products have gone through clinical trials and released to the market. For example, carrot-derived ElelysoTM (taliglucerase alfa) (<http://www.elelyso.com/>), a medicine for Type 1 Gaucher disease, was approved by FDA and marketed in the U.S by Pfizer Company in 2012 (Stoger et al. 2014). A tobacco-derived humanized antibody cocktail ZMapp has been successfully used for the treatment of Ebola virus infected humans in the 2014 West Africa Ebola outbreak (Geisbert 2014; Qiu et al. 2014).

In this study, the rNP gene of H3N2 swine influenza virus was expressed in maize seed using a maize endosperm-specific promoter. Maize seed was chosen because it is one of the components of swine feed. Maize seed is also a stable organ for protein storage. Previous work has shown that maize-derived antigens could elicit an immune response and protect animals from diseases (Chikwamba et al. 2002). We generated 12 transgenic maize lines expressing rNP in seeds. Enzyme-linked immunosorbent assay (ELISA) of the T1 seeds showed that the accumulation of NP protein ranged from 8.0 to 35 µg of NP/g of maize seed. Oral administration of maize-derived rNP (MNP) elicited NP-specific antibodies in mice. The antibody titer induced in the group receiving MNP was comparable to the control groups receiving Vero cell-derived NP (VNP) or alphavirus replicon particle-derived NP (ANP), suggesting that MNP is immunogenic. Our study shows that the plant is a good system to produce vaccines.

Materials and methods

DNA construct

The nucleoprotein (NP) gene of Influenza A Virus H3N2 (gene bank accession CY157826.1) was codon optimized and synthesized by GeneArt[®] (Life Technologies, Grand Island, NY, USA). The endosperm-specific promoter of the maize 27-kDa γ -zein gene was chosen to drive the expression of NP protein in transgenic maize seeds. The vector construct of rNP expression in maize seed endosperm is depicted in Fig. 1. Maize γ -zein signal peptide

(Marks et al. 1985) was added upstream of the NP gene. C-terminal SEKDEL (ER-retention signal) (Munro and Pelham 1987) and VSP (soybean vegetative storage protein) terminator (Mason et al. 1993) were added downstream of the NP gene, respectively. A complete NP DNA cassette with *Pst*I-*Eco*RI sites was inserted in multiple cloning sites of a binary vector pTF101.1 (Paz et al. 2004), resulting in construct pHN05 (Fig. 1a). The binary vector pTF101.1 contains an herbicide resistant gene (*bar*) that can be used as selectable marker for maize transformation. The construct pHN05 was transferred into *Agrobacterium tumefaciens* strain EHA101 (Hood et al. 1986) prior to maize transformation.

Maize transformation

Agrobacterium-mediated plant transformation was conducted by the Plant Transformation Facility of Iowa State University. Maize Hi-II immature embryos were infected with *A. tumefaciens* EHA101 (Frame et al. 2002) harboring the pHN05 plasmid. Screening of the putative transgenic events was performed using the herbicide, Bialaphos. Transgenic plants were generated and advanced in the

greenhouse to obtain T1 seeds. NP transgenic maize was self-pollinated or cross-pollinated with B73 for seed propagation.

Southern blot analysis

Southern blot analysis was carried out to estimate copy numbers of the NP transgene integration in the transgenic maize plant genome. Genomic DNA (10 µg) isolated from young leaves of T1 plants (Doyle and Doyle 1990) was digested overnight with *Hind*III enzyme. The digestion products were fractionated in 0.8 % agarose gel then cross-linked into a membrane (Ambion® *BrightStar*®-Plus Positively Charged Nylon Membrane) using a cross-linker apparatus. The membrane was pre-hybridized in Church's buffer for 2 h at 65 °C before adding the probe. An NP-specific probe was amplified from pHN05 plasmid by PCR using forward (5' AAGGGCAAGTTCCAGACAGC 3') and reverse (5' CGGACAGCTCGAACACGC 3') primer pair (Fig. 1) then labeled with ³²P dCTP. The probe was purified then denatured at 90 °C for 10 min prior to overnight membrane hybridization at 65 °C. The membrane was washed 4 times with high stringency washing solution (0.2 × SSC/saline-sodium citrate buffer, 0.1 % SDS/sodium dodecyl sulfate). The film was developed after 16 h exposure at −80 °C.

RNA isolation and RT-PCR

Transcription level of the NP gene was analyzed using the reverse-transcriptase PCR (RT-PCR) method. Total RNA (from maize leaf, seed, silk, husk) was extracted using Qiagen RNeasy Plant Mini kit (Qiagen Inc, Valencia, CA, USA) following supplier's instructions. An aliquot (1 µg) of the total RNA was used as template to synthesize complementary DNA (cDNA) using SuperScript™ II reverse transcriptase (Invitrogen, Grand Island, NY, USA) in a total reaction of 20 µl. NP transcripts (±450 bp) were amplified from 2 µl of cDNA (100 ng) using forward (5' AAGGGCAAGTTCCAGACAGC 3') and reverse (5' AGAGTCCATG GCCTCCACGT 3') primer pair. Actin primers forward (5' ATTCAGGTGATGGTGTGAGCCACAC 3') and reverse (5' GCCACCGATCCAGACACTGTACTTCC 3') were used to amplify actin as internal control of house-keeping gene. Equal amounts of PCR product were loaded into each well and transcription level was determined semi-quantitatively.

Protein extraction and protein concentration determination

Total soluble protein was extracted from maize kernels for NP protein expression analysis. A single maize kernel was

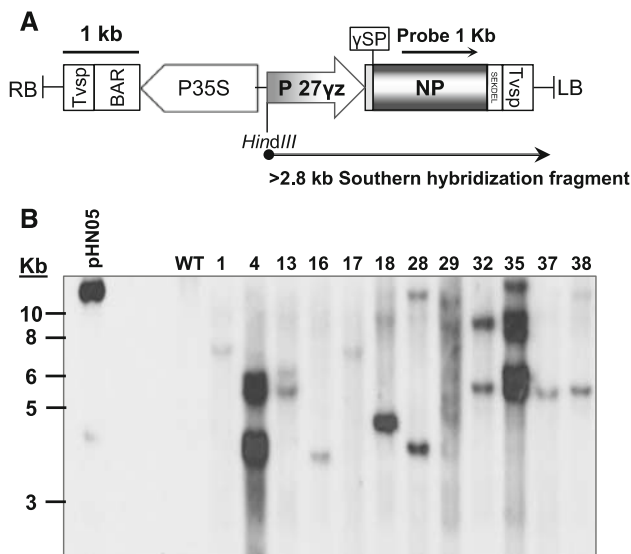


Fig. 1 **a** Schematic diagram of the T-DNA region in vector pHN05. The codon-optimized rNP gene driven by the 27-kDa γ -zein promoter (P27 γ z) was transformed into maize immature embryos via *Agrobacterium*-mediated transformation. γ SP maize γ -zein signal peptide, SEKDEL endoplasmic reticulum retention signal, TVSP soybean vegetative storage protein gene terminator, *bar* bialaphos resistance gene, P35S the cauliflower mosaic virus 35S promoter, LB *Agrobacterium* T-DNA Left border, RB *Agrobacterium* T-DNA right border, *Hind*III restriction enzymes, kb kilobases. **b** Southern blot analysis of twelve rNP transgenic maize lines. Genomic DNA was digested with *Hind*III, separated, and then transferred onto a nitrocellulose membrane before being hybridized with an rNP probe. pHN05 rNP construct plasmid as positive control, WT non-transgenic B73 inbred line as negative control, kb kilobases

ground into powder using the Genogrinder 2,000 operated at 550 stroke/min for 90 s at the Iowa State University Plant Tissue and Seed Grinding Facility. The sample was homogenized in 10 μ l of sodium phosphate buffer (pH 6.6) supplemented with a cocktail of protease inhibitors per mg of maize powder as described (Moeller et al. 2009). The sample was incubated by shaking at 37 °C, 250 rpm for 2 h then centrifuged in a tabletop microcentrifuge at 13,000 rpm for 20 min. The supernatant was transferred into a new tube. Total soluble protein (TSP) concentration was quantified with the Bradford (1976) method using BSA (bovine serum albumin) as standard. The protein extract was used for NP quantification by ELISA (Enzyme-linked immunosorbent assay), Western blot and for animal experiments.

Western blot

Maize protein extract (20 μ g) was boiled for 5 min then directly put on ice (Laemmli 1970). Samples were loaded into a 4–15 % SDS gel (Bio-Rad, Hercules, CA, USA) run at 110 volts for 90 min. Western blot was carried out at room temperature unless otherwise stated. The gel was transferred onto a 0.2- μ m nitrocellulose membrane using a semi-dry blot transfer apparatus (Bio-Rad, Hercules, CA, USA) following the manufacturer's instructions. Non-specific binding was blocked by incubating the membrane with 5 % non-fat dry milk in 1X phosphate-buffered saline (1X PBS: 137 mM NaCl, 10 mM Na₂HPO₄, 1.76 mM KH₂PO₄, pH 7.4) for 2 h. The membrane was washed 4 times with 1X PBST [PBS-0.05 % Tween-20 (v/v)] then incubated with a 1:1,000 dilution of mouse anti-NP monoclonal antibody (Milipore, Billerica, MA, USA). Secondary antibody goat anti-mouse-IgG conjugated with horseradish peroxidase (HRP) (Sigma Aldrich, St. Louis, MO, USA) in a 1:5,000, dilution was added to the membrane followed by 4 times washing with 1X PBST. The membrane was incubated with Sure Blue 3,3',5,5'-tetramethylbenzidine (TMB) substrate (KPL, Gaithersburg, MD, USA) for 20 min at room temperature for protein visualization.

NP quantification

Nucleoprotein antigen concentration in transgenic maize seed was quantified using an ELISA method. Eight PCR positive seeds from each transgenic line were tested. Total protein extracts (50 μ l/well) diluted in 1X PBS buffer were added into a 96-well plate. Serial dilution of pure recombinant NP (NBP2-26531, Imgenex, San Diego, CA, USA) was used as a standard. Analysis of samples and standards was carried out in duplicate. The plate was incubated overnight at 4 °C followed by 3 times washing with 1X PBST buffer. Blocking solution (5 % non-fat dry milk in

1X PBS) was added to each well to remove non-specific binding then the plate was incubated at 37 °C for an hour. After washing 3 times using 1X PBST, samples were incubated with mouse anti-NP antibody (1:1,000) at 37 °C for 1 h. After washing, samples were incubated with the goat anti-mouse secondary antibody (1:5,000, 50 μ l/well) at 37 °C for 1 h. TMB substrate was added to each well and absorbance was measured using a plate reader at 405 nm. NP concentration was determined based on known concentration of standards.

Pig study

Four experimental crossbred pigs (approximately 7 weeks old) were obtained from a commercial herd (Wilson's Prairie View Farms, Burlington, WI, USA) with no serological evidence of influenza infection, and were randomly assigned into two groups consisting of two pigs per group. Animals were immunized with 16 μ g MNP or non-transgenic maize (NTM) extract. Protein extract was mixed with adjuvant (Benchmark BioLabs item #70101) at a 4:1 ratio in a 5 ml final volume. Immunization (5 ml) was administered intramuscularly in the neck. Each pig received prime and boost immunization at days 0 and 21, respectively. Sera samples were collected at the day of prime (day 0), day of boost (day 21) and 6 days after boost immunization (day 27) for NP-specific antibody detection. The pig study was approved by the Institutional Animal Care and Use Committee of Harrisvaccines, Inc.

Mouse study

Female BALB/c mice (6–8 weeks old) were purchased from Charles River Laboratories International Inc. (Willington, MA, USA). The experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of Iowa State University. All mice were maintained in the Iowa State University animal facility. Mice were housed in plastic cages (4 mice per cage) at 20 °C with reverse 12 h light/dark cycle. The animals were allowed to a 2-week acclimation before the experiment started. Bedding was changed twice per week and mice had ad libitum access to feed and water. Body weight was recorded every week.

Mouse immunization

Three groups consisting of 8 mice per group were vaccinated with 20 μ g of MNP, VNP (NP expressed in Vero cells), or non-transgenic maize protein extracts. For positive control, 4 mice per group received ANP (alphavirus replicon particles expressing NP) at a dosage 5×10^6 of replicon particles (RP)/100 μ l; for negative control, the

same number of mice received the same volume and concentration of AGFP (alphavirus replicon particles expressing green fluorescent protein) (Vander Veen et al. 2013). All animals received three immunizations that were given in prime-boost design as summarized in Table 1. During prime immunization (day 0), immunogens (except groups receiving ANP and AGFP) were administered subcutaneously with the adjuvant Imject Alum (Pierce, Rockford, IL, USA) in 1:1 (v:v) ratio. Boost immunizations (days 21 and 42) were administered orally through gavage without adjuvant. Prime and boost immunizations of groups receiving ANP and AGFP were administered subcutaneously without adjuvant. Mice were fasted 12 h prior to oral boost until 30 min after immunization.

Mouse specimen collection

Blood and fecal pellets were collected one day prior and 1 week after each prime or boost immunization. Serum was separated from other blood cellular components by centrifugation at 12,000 rpm and stored at -20°C until the day of analysis. Fecal pellets were frozen and lyophilized for 48 h prior to antibody analysis. One week after the last boost, mice were euthanized and blood and spleens were collected. Spleens were dissociated to a single cell suspension in Roswell Park Memorial Institute (RPMI) 1640 complete media (GIBCO, Grand Island, NY, USA) supplemented with 2 mM glutamine, 25 mM of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer, 50 $\mu\text{g}/\text{ml}$ gentamicin, and 10 % heated-inactivated fetal bovine serum (FBS) (Zhai et al. 2007).

Antibody detection

Antibodies specific to NP were analyzed by an NP ELISA (IDEXX Laboratories, Inc, Westbrook, Maine) that is based on a competitive ELISA (Ciacci-Zanella et al. 2010). The test was performed by the Veterinary Diagnostic Laboratory of Iowa State University. The mouse serum

sample was added onto an NP-coated plate followed by washing. A known horseradish peroxidase-conjugated antibody specific for NP was added followed by washing. TMB substrate was added and the absorbance was measured. The amount of antibodies present in the unknown sample is inversely proportional to the absorbance reading, due to competitive inhibition of known conjugate binding. Test results are presented as S/N values (sample to negative control ratio). The cutoff value for positive samples in mouse is $S/N \leq 0.67$ while the cutoff value for swine sample is $S/N \leq 0.70$. Serum and fecal samples were analyzed for antibody isotypes using ELISA. Fecal extraction was conducted by adding 10 μl 1X PBS supplemented with protease inhibitor cocktail (Sigma Aldrich, St. Louis, MO, USA) per mg of dry fecal sample. Samples were incubated with vigorous shaking at 9°C overnight then centrifuged at 12,000 rpm for 15 min. A 96-well ELISA plate was coated with 10 $\mu\text{g}/\text{ml}$ rNP (Imgenex, San Diego, CA, USA) in carbonate buffer pH 9.6 and incubated overnight at 4°C . After washing with 1X PBST, the plate was blocked with 5 % bovine serum albumin (BSA) in 1X PBS then washed. A serum sample diluted 1:50 was added to each well and the plate was incubated at 37°C for 2 h followed by washing with 1X PBST. Goat anti-mouse (IgA, IgG, IgG1, or IgG2a) peroxidase-conjugated antibody was added and incubated at 37°C for 1 h followed by washing. TMB substrate was added for color development. Reaction was stopped by adding 1 N HCl then the absorbance was read at 450 nm.

Enzyme-linked immunospot (ELISPOT)

Red blood cells in mouse splenocytes were lysed by adding 1X flow cytometry mouse lyse buffer (R&D Systems, Inc., Minneapolis, MN, USA). Samples were submitted to the Cell Hybridoma Facility of Iowa State University for cell counting using flow cytometry. Cells were used to measure mouse NP antigen-specific IL-4 and interferon gamma production using ELISPOT. Splenocytes cells ($1 \times 10^6/\text{ml}$)

Table 1 Design of immunization

Immunogens	# Mice	Prime/day 0 (immunogen + adjuvant ^a)		Boost/day 21 and 42 (without adjuvant)	
		Route	Dose	Route	Dose
MNP	8	Subcutaneous	20 μg	Gavage	20 μg
VNP	8	Subcutaneous	20 μg	Gavage	20 μg
NTM	8	Subcutaneous	20 μg	Gavage	20 μg
ANP	4	Subcutaneous ^b	5×10^6 RP	Subcutaneous	5×10^6 RP
AGFP	4	Subcutaneous ^b	5×10^6 RP	Subcutaneous	5×10^6 RP

RP replicon particles

^a Adjuvant (aluminum hydroxide) was added in 1:1 ratio respective to the volume of immunogen

^b Prime immunization was administered without adjuvant

were added to each well in a 96-well plate. The procedure was performed following manufacturer's instruction (R&D Systems, Inc., Minneapolis, MN, USA). Cells were stimulated with two swine influenza virus strains (2009 pandemic strain H1N1 and H3N2) overnight at 37 °C in a CO₂ incubator. After washing with 1X PBST, biotinylated antibody specific to IL-4 or IFN- γ was added subsequently. The plates were incubated overnight at 4 °C followed by washing 3 times with 1X PBST. Streptavidin-AP was added to the well after incubation and washing, 5-bromo-4-chloro-3'-indolylphosphate p-toluidine salt (BCIP)/nitro-blue tetrazolium chloride (NBT) chromogenic substrate was added for development of spots. Chromogen solution was discarded and microplates were dried completely before counting ELISpots under the microscope.

Statistical analysis

Statistical analysis was performed using JMP 10 software (SAS Institute Inc., Cary, NC, USA). Data were analyzed using analysis of variance. Significance was set at *P* value ≤ 0.05 .

Results and discussion

Stable expression of rNP in transgenic maize plants

In this study, the H3N2 Nucleoprotein gene (referred to as rNP) expression was driven by the maize endosperm-specific promoter, 27-kDa γ -zein (Marks et al. 1985) that facilitates the accumulation of protein in seed endosperm. This strategy was chosen to confine the transgene expression to a specific compartment and thus prevent proteolytic degradation (Lee et al. 2014). Seed expression can also reduce the risk of contamination of the environment compared to the expression in leaves or roots (Ou et al. 2014). An endoplasmic reticulum (ER) targeting signal, SEKDEL was fused in the C-terminus of the rNP gene that functions to retain the translated proteins inside the ER (Ellgaard and Helenius 2003).

The NP gene used in this study was codon optimized and synthesized for mammalian cell expression (matched the sequence expressed by Vero cell, kindly provided by Dr. Ryan Vander Veen). The GC percentage of this gene is around 63 %. We reasoned that this version would be appropriate for maize expression since maize has high GC percentage (around 55 %) (<http://www.kazusa.or.jp/codon/>). Construct pHN05 containing the codon-optimized rNP and selectable marker *bar* genes (Fig. 1a) was transformed into the maize genome through *Agrobacterium*-mediated transformation. Twelve transgenic lines were generated then subjected to Southern blot assay for gene integration and

copy number analysis. Genomic DNA was digested with *Hind*III enzyme that recognized one site in the promoter region of the pHN05 construct. The number of bands appearing on the hybridization film suggests copy number of rNP genes that are integrated into the maize genome. The intact NP gene will demonstrate a band size larger than 2.8 Kb and bands of lower molecular weights are likely partial integration of the NP construct into maize genome. Eight out of 12 lines (lines 4, 13, 18, 28, 29, 32, 35, and 38) had multiple copies of rNP genes as shown by the presence of multiple bands (Fig. 1b) and four lines (1, 16, 17 and 37) showed one hybridization band, indicating a single integration of the rNP gene. Those twelve lines were evaluated for rNP protein accumulation. Three events with the highest rNP accumulation were advanced for production of seeds for five generations (T5). Transgenic plants showed similar phenotype compared to wild-type plants and no abnormal growth.

Analysis of rNP protein accumulation in transgenic maize seeds

First-generation (T1) transgenic maize seed was analyzed for rNP protein accumulation with an end-point ELISA assay. The concentration of rNP protein was determined against known concentrations of purified commercial NP protein (Imgenex, San Diego, CA, USA). This experiment was carried out to screen lines that have high expression of NP protein. The accumulation of rNP protein in the T1 transgenic maize seeds ranged between 8.0 and 35 $\mu\text{g/g}$ corn seed (Fig. 2). Lines 1, 16 and 18 showed high rNP protein accumulation with the concentration 35.0, 32.7 and 29.2 $\mu\text{g/g}$ corn seed, respectively. Those lines were advanced in the green house for seed productions. Analysis of NP expression in T3 seeds of line 1 that had a single copy of rNP showed an increase of NP accumulation up to 70 $\mu\text{g/g}$ of corn seed.

The rNP protein accumulation did not appear to correlate with the transgene copy number. In this study, lines 1 and 16 had high rNP expression levels of 35.0 and 32.7 $\mu\text{g/g}$, respectively. However, single copy events 17 and 37 showed low (9.7 $\mu\text{g/g}$) and moderate (18.7 $\mu\text{g/g}$) rNP expression, respectively (Fig. 2). On the other hand, multiple copies event 18 had similar high gene expression (29.2 $\mu\text{g/g}$) compared to the single copy Line 1. Multiple copy numbers of transgenes could lead to the increase of gene expression but it could also result in gene silencing (Flavell 1994; Stam et al. 1997; Hood et al. 2003; Schubert et al. 2004).

Seed-specific expression of rNP transgenic maize

Molecular analysis was performed to confirm rNP expression in transgenic maize seeds. Different tissues such

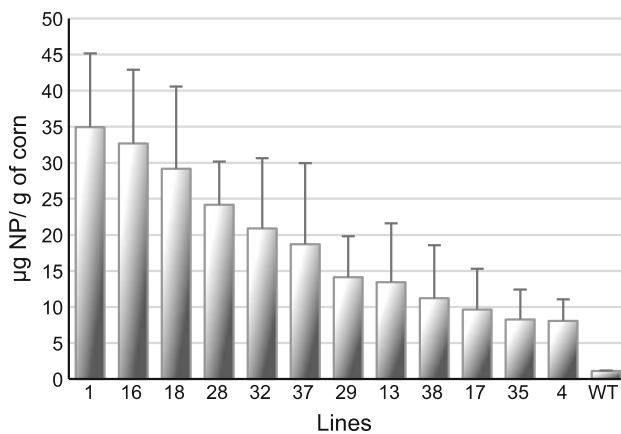


Fig. 2 rNP accumulation in T1 transgenic maize. Eight PCR positive seeds from each line were analyzed by ELISA. Three lines (1, 16, and 18) were advanced in the green house for seed production. Error bars represent the standard deviation of eight biological replicates

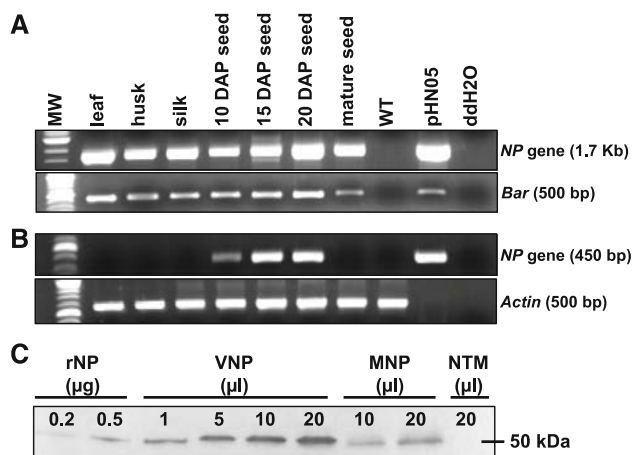


Fig. 3 rNP expression analysis in transgenic maize (*line 1*). **a** PCR analysis for NP gene detection; **b** RT-PCR analysis for RNA detection; **c** western blot analysis for maize-derived rNP protein detection. *Bar* gene primers are used as a positive control for PCR analysis. *Actin* gene primers are utilized as a positive control for transcript analysis. rNP (NBP2-26531) from Imgenex is used as positive control for protein analysis. *MW* molecular weight marker, *WT* wild-type B73 maize, *pHN05* rNP construct plasmid control, *DAP* days after pollination. *rNP* recombinant NP, *VNP* Vero cell-derived NP, *MNP* maize-derived rNP, *NTM* non-transgenic maize

as leaf, husk, silk and seed were collected from transgenic line 1. PCR analysis showed amplification of both rNP and *bar* genes in transgenic maize (Fig. 3a). Reverse-transcriptase PCR (RT-PCR) analysis showed that rNP transcripts were mainly accumulated in seed and the level increased as the seed matured (Fig. 3b). The rNP gene was driven by the maize 27-kDa γ -zein promoter, and this transcription level pattern is in agreement with the pattern of maize zein transcript accumulation during maize seed development where transcripts reach their peak during

15–25 days after pollination. Transcripts decrease when seeds reach the mature stage (Russell and Fromm 1997).

High accumulation of rNP in the seed indicates proper activity of the endosperm-specific promoter and suggests that a tissue-specific promoter is an efficient strategy for recombinant protein accumulation. Seed is a stable organ for recombinant protein accumulation and it is a natural component of animal feed (Ma et al. 2003; Giddings et al. 2000). Proteins accumulated in seed are still functional and available even years after harvesting (Giddings et al. 2000; Ma et al. 2003; Moravec et al. 2007; Wu et al. 2007).

NP protein is a non-glycosylated protein (Townsend et al. 1985) and prediction with NetNGlyc 1.0 server showed that no potential glycosylation site was found in the rNP sequence (<http://www.cbs.dtu.dk/services/NetNGlyc/>) (Gupta et al. 2004). Glycosylation should be considered before expressing antigens or antibodies in plants. This is important due to different glycosylation patterns between animal and plant cells. Improper glycosylation patterns may lead to incorrect protein folding that could result in a non-functional or unstable protein (Twyman et al. 2012).

Western blot analysis of maize-derived rNP was carried out to confirm that a correctly folded protein was accumulated in the transgenic plants. Membrane visualization showed similar banding pattern of 55 kDa between pure NP protein, VNP and MNP (Fig. 3c). This result indicates that proper NP protein was expressed in maize seeds. The highest rNP expression observed in this study was about 0.06 % TSP (total soluble protein). A previous study showed similar results in which highest expression of LT-B vaccine driven by maize 27-kDa γ -zein promoter was 0.07 % (Chikwamba et al. 2002). A recent study showed high accumulation of the hepatitis B surface antigen (HBsAg) in maize seed driven by an improved version of globulin 1 promoter showing a production level of 0.51 % TSP (Hayden et al. 2012). Strategies are important when designing construct to express foreign protein in seed. The choice of promoter, signal sequence for organelle targeting, and terminator can contribute to the expression level of the transgene (Orellana-Escobedo et al. 2014).

Animal study

In an attempt to prove that maize-derived NP is immunogenic, we conducted a preliminary study in pigs. Sera samples collected during the experiment were sent to the Veterinary Diagnostic Laboratory of Iowa State University for NP-specific antibody analysis. As can be seen in Fig. 4, there was no NP-specific antibody detected in all experimental pigs prior to prime immunization (day 0) and 21 days after the first vaccination (day of boost). Significant immune response was induced by MNP

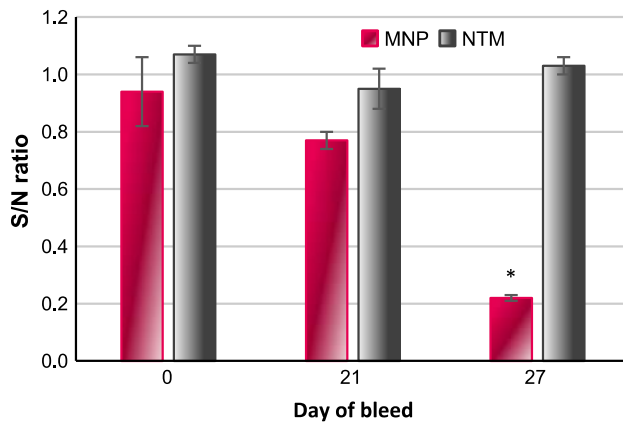


Fig. 4 Anti-NP antibody serum analysis in experimental pigs after MNP administration. Two pigs were injected with 16 μ g of MNP or NTM extract. Day 0, prime immunization; day 21, booster vaccination; day 27, 6 days post booster vaccination. *NTM* non-transgenic maize, *MNP* maize-derived rNP. *S/N* (sample/negative) response is the ratio of the sample optical density (OD_{650}) reading to the kit negative control OD reading. Asterisk indicates significantly different ($P < 0.05$) by Student's *t* test. Error bars represent the standard deviation of the mean of biological replicates

administration (intramuscular injection) 6 days after boost immunization (day 27) (Fig. 4). NP-specific antibody analysis showed positive response only in the group receiving MNP while the negative control group remained seronegative during the entire experiment, suggesting that maize-derived NP is immunogenic.

Our goal was to develop an orally delivered vaccine for influenza. Due to resource limitations, further studies were performed using laboratory mice as a model. A mouse study was designed to evaluate MNP as an orally fed vaccine for influenza A virus (IAV). In a preliminary study, mice were fed maize pellets containing 20 μ g of MNP, 20 μ g of VNP mixed with non-transgenic maize powder, or non-transgenic maize (NTM). Maize pellets in the MNP and VNP groups were supplemented with or without 20 μ g of MLTB (maize-derived LT-B) as adjuvant (Chikwamba et al. 2002). A positive control group was administered a subcutaneous injection of 20 μ g VNP. A negative control group was immunized subcutaneously with phosphate-buffered saline. Oral feeding was administered 3 times at 3 week intervals. Sera and fecal analyses to detect NP-specific antibodies did not show an immune response induced by NP oral feeding. Sera analysis showed positive result only in the positive control group that received the subcutaneous injection of VNP (Fig. S1, supplemental materials). We speculated that oral feeding was not an effective route for vaccine administration.

To investigate whether the negative immune response was due to ineffectiveness of MNP or the delivery method, we carried out a second mouse study in which MNP was administered orally through gavage and subcutaneously. In

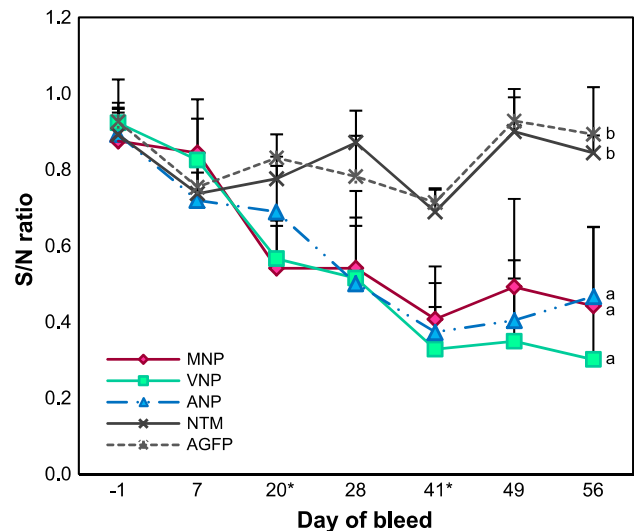


Fig. 5 Oral administration of MNP induces NP-specific antibodies in experimental mouse serum. *MNP* maize-derived rNP, *VNP* Vero cell-derived NP, *ANP* alphavirus replicon particle-derived NP, *NTM* non-transgenic maize and *AGFP* alphavirus replicon particles expressing green fluorescent protein. *S/N* (sample/negative) response is the ratio of the sample optical density (OD_{650}) reading to the kit negative control OD reading. The values with different superscripts are significantly different ($P < 0.05$) by Student's *t* test. Error bars represent the standard deviation of mean of biological replicates. Asterisk indicates the day before boost immunization

this study, immunization was conducted in a prime-boost design, in which immunizations were 3 weeks apart. Prime immunization was administered subcutaneously with adjuvant at a ratio of 1:1 (v/v). Each mouse then received two boost vaccinations that were administered through gavage (except boosts to ANP and AGFP groups were administered subcutaneously). As can be seen in Fig. 5, NP-specific antibody in MNP fed group showed a high antibody titer that is comparable to the VNP group and positive control ANP group. Specific antibodies were detected after boost vaccination and remained high until the day of termination. This result suggested that maize-derived rNP (MNP) was able to induce equivalent humoral immune response compared to animal-derived rNP (VNP or ANP). Our result is in agreement with observations reported by Huang et al. (2012), in which comparable humoral immune response could be induced by rNP expressed in both prokaryotic and eukaryotic systems.

Further antibody analyses (IgG and IgA) were performed to detect immune responses elicited by MNP oral administration in mouse. As shown in Fig. 6a, similar levels of IgG can be detected from the mice groups treated with either MNP, VNP or ANP. Antibody isotype analyses showed that IgG2a antibody titer was higher than IgG1, suggesting that IgG2a was highly induced in this experiment (Fig. 6b, c). Production of IgG2a is stimulated by interferon gamma ($IFN-\gamma$), which indicates cell-mediated

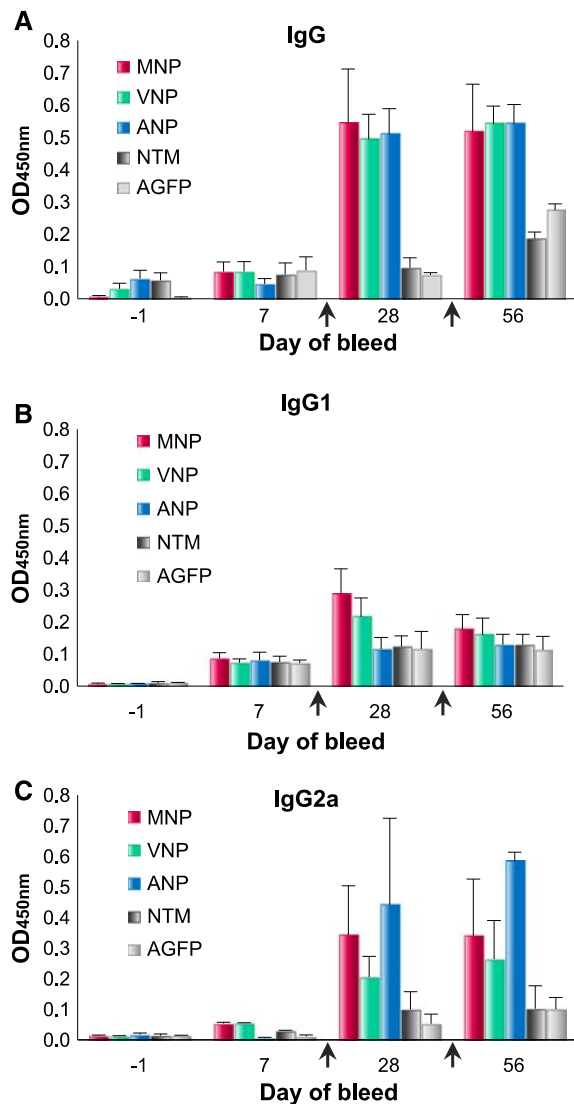


Fig. 6 Immunoglobulin (IgG) antibody analysis in orally immunized mice. **a** anti-NP IgG levels, **b** anti-NP IgG1 levels, **c** anti-NP IgG2a levels in mouse sera after MNP oral administration. MNP maize-derived rNP, VNP Vero cell-derived NP, ANP alphavirus replicon particle-derived NP, NTM non-transgenic maize and AGFP alphavirus replicon particles expressing green fluorescent protein. Error bars represent the standard deviation of the mean of biological replicates (see Table 1 for number of replications). Arrow indicates the day of boost immunizations (day 21 and 42)

immunity (Pertmer et al. 1996). In this study, we performed oral gavage administration with an attempt to induce a mucosal immune response. However, no notable increase of IgA in any group could be detected in our study, suggesting poor mucosal immune response to the NP regardless of the source of production.

To further confirm immune responses induced by MNP oral administration, cytokine analysis using the ELISPOT assay was performed. Both interleukin-4 (IL-4) and interferon gamma (IFN- γ) cytokines that indicate TH2 and TH1

Table 2 Cytokine IL-4 production in orally immunized mice

Groups	H1N1	H3N2
MNP	12.75 \pm 7.67 ^a	12.13 \pm 6.47 ^a
VNP	31.19 \pm 13.48 ^b	34.31 \pm 14.20 ^b
ANP	30.25 \pm 7.60 ^b	28.63 \pm 6.29 ^b
NTM	3.11 \pm 1.19 ^c	3.17 \pm 1.30 ^c
AGFP	3.88 \pm 1.11 ^c	5.25 \pm 0.96 ^c

Mean IL-4 secreting cells per 1×10^6 mouse splenocytes at day 56. The values with different superscripts within a column are significantly different ($P < 0.05$) by Student's *t* test

mediated immune responses, respectively, were analyzed. Splenocytes were stimulated against two influenza virus strains (H1N1 and H3N2) to test heterosubtypic responses induced by MNP. The results are summarized in Table 2. Cytokine IL-4 was highly induced in the groups that received VNP and ANP, which is significantly different compared to other groups ($P < 0.05$). A moderate IL-4 response was detected in the MNP group, although not as high as the positive control groups (VNP and ANP) ($P < 0.05$). However, the result showed that the MNP group is significantly different compared to the negative groups that received non-transgenic corn (NTM) and AGFP ($P < 0.05$), both of which had undetectable IL-4. The numbers of IL-4 spots induced by pandemic H1N1 and H3N2 in all groups were similar ($P < 0.05$). This result confirmed that MNP induces a humoral response against different strains of influenza viruses, suggesting that NP is a conserved protein. Interestingly, we could not detect a cytokine IFN- γ response in any groups except for the ANP group (too many to count) using the ELISPOT assay despite previous result that showed IgG2a antibody was highly induced. This discrepancy could be caused by the IFN- γ independent production of IgG2a (Markine-Goriaynoff et al. 2000). ANP was shown previously (Vander Veen et al. 2013) to induce TH2 and TH1 mediated immune responses. Alphavirus replicon particle vaccine mimics the wild-type virus infection, except it does not have the structural protein that can cause systemic infection. It is possible that the dose of rNP used in this study was too low to induce the production of IFN- γ . A study by Huang et al. (2012) showed that intramuscular injection of *E. coli*-derived NP induced the production of IFN- γ in mice that were administered with 90 μ g of NP but not in the groups receiving 10 or 30 μ g of NP. Further study testing different doses of MNP will confirm this speculation.

An ideal vaccine should be immunogenic, inducing both humoral, mucosal and cell-mediated immune (CMI) responses. Our study showed that the amount of MNP used induced a humoral, but not a TH1 CMI response. Various forms of NP vaccinations and their immune responses have

been reported. They include live attenuated *Salmonella* vaccine, DNA, virus vector or purified protein (Ashraf et al. 2011; Pertmer et al. 1996; Kim et al. 2013; Huang et al. 2012) in which both humoral and CMI responses were induced. DNA or virus-based vector vaccinations are considered to mimic live virus infection that can induce the major histocompatibility complex class I presentation (Cohen et al. 1998; Lambert and Fauci 2010). A mucosal immune response is also an important factor for vaccine design as the first defense against viral infection that relies on the route of vaccination (Rose et al. 2012). Intramuscular immunization of NP protein did not induce a mucosal response (Sanchez et al. 2014), but intranasal administration was able to induce mucosal immunity (Luo et al. 2012). Furthermore, adjuvant is also important to elicit both humoral and CMI responses of NP-based vaccine (Sanchez et al. 2014; Wang et al. 2014).

In the current study, we did not observe a CMI response. The relative importance of CMI or antibody to NP in protection is somewhat controversial. CMI to NP is thought to be important because protection is possibly conferred via CD8⁺ T cells (Epstein et al. 2005; Jimenez et al. 2007; Kreijtz et al. 2008; Zhou et al. 2010). Others have suggested that antibodies against NP are important for protection and that immune serum alone can transfer protection (Carragher et al. 2008; Eliasson et al. 2008; LaMere et al. 2011a, b).

Conclusion

Influenza A virus infection causes severe respiratory disease. However, the development of a vaccine for this disease is a complex process due to the antigenic shift and drift of the virus. This leads to a desired universal vaccine that is based on conserved proteins. A universal vaccine is expected to provide some level of protection against influenza virus infection before the strain-specific vaccine is produced and administered.

Our study provides evidence of the accumulation of a universal vaccine protein candidate in a plant. Oral administration of plant-derived rNP induced antibody production but not cell-mediated immunity in mice. Few factors such as route of delivery, dose and adjuvant are important for the immune response induction. Further study combining these factors will be useful for the improvement of NP vaccine efficacy as a universal influenza vaccine.

Author contribution statement KW and BB conceived research. KW, BB and HN designed experiments. HN conducted all experiments except for pig experiment. BB and JC were also involved in mouse experiments. MM

conducted pig experiment. BB, MM and JC contributed materials and expertise. HN and KW analyzed data and wrote the manuscript. All the authors read and approved the manuscript.

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Conflict of interest HN, BB, JC and KW declare that they have no conflict of interest. MM is an employee of Harrisvaccines, Inc., which provided materials and expertise for the work. However, this does not alter the author's adherence to all the Plant Cell Reports policies on sharing data and materials.

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