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Development of a candidate vaccine for *Newcastle disease virus* by epitope display in the *Cucumber mosaic virus* capsid protein

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Abstract

A peptide fusion to the capsid protein (CP) of *Cucumber mosaic virus* (CMV) was designed to express either a 17 amino acid (aa) neutralizing epitope of the *Newcastle disease virus* (NDV) fusion (F) protein or an eight aa neutralizing epitope of the NDV hemagglutinin-neuraminidase (HN) protein. Fusions of the F, HN and HN2 (duplicated HN epitope) were made in the internal βH-βI loop (motif 5) within the CMV CP. Recombinant RNA3 transcripts of the Ixora strain of CMV were inoculated on to *Nicotiana benthamiana*, together with CMV RNA1 and CMV RNA2. When the F and HN epitopes were placed in the internal motif, the modified virus was infectious and the HN NDV epitope was recognized by anti-NDV sera. However, in some plants, deletions of one to several of the inserted amino acids occurred. A duplication of the HN epitope rendered the virus non-viable.

Introduction

Cucumber mosaic virus (CMV), the type member of the genus *Cucumovirus* in the family *Bromoviridae*, has one of the widest host ranges among plant viruses, making it an attractive candidate as a viral-based transient expression vector for production of edible vaccines in plants. Its genome is divided and consists of three (+)-sense, single-stranded genomic RNAs (RNAs 1–3) and a subgenomic RNA (RNA4) which is generated by transcription from RNA3 during infection and serves as the messenger RNA for the single 24 kDa viral capsid protein (CP). The complete sequence of the Ixora strain of CMV is known and *in vitro*-derived transcripts are infectious (Tousignant *et al.* 1996). The viral genome is encapsidated by an icosohedral $T = 3$ protein shell with ~180 copies of the CP. Purified virus preparations contain four RNA species housed in three types of particles containing either RNA1, RNA2, or RNA3 + RNA4, and each with a size of 25–28 nm diameter. The crystallographic structure of CMV has been determined (Smith *et al.* 2000), and the βH-βI loop (amino acid positions 190–198) is a highly conserved, negatively charged, and antigenic, playing an essential role in aphid transmission (He *et al.* 1998, Liu *et al.* 2002).

Newcastle disease virus (NDV), a member of family *Paramyxoviridae*, genus *Avulavirus,* infects many species of both domestic and wild birds, with the mortality and morbidity rates varying among species and strains of the virus (Alexander 1988). The most widely used vaccines against NDV are live viruses that can themselves cause mild disease. NDV virions are enveloped and

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contain a single molecule of linear, negative sense RNA. The lipid envelope contains two transmembrane glycoproteins, which form spike-like structures on the surface of the particle: the fusion protein (F) and the hemagglutinin-neuraminidase (HN) protein. Virus infection is initiated by the action of these two proteins: HN mediates the attachment of the virus to a host cell receptor; F mediates virus penetration into the host cell and virus-induced cell fusion and hemolysis. These two surface proteins are important targets for the host immune response. Antibodies to the F protein appear to be important for preventing infection and spreading of the virus *in vivo*. Major antigenic determinants and epitopes that stimulate the production of virus-neutralizing antibodies have been determined for the F (Toyoda *et al.* 1988) and HN proteins (Chambers *et al.* 1988).

Our goal was to develop an alternative anti-NDV vaccine candidate that is subunit-based, cost-effective and conveniently produced in a plant system. To achieve this, we have chosen a transient expression technology where high-level production of desired foreign proteins in the host plant results from the rapidly amplified genome of an infectious plant virus. A presentation system based on the coat protein (CP) of *Cucumber mosaic virus* as a display carrier was designed to express either the 17 amino acid (aa) neutralizing epitope (antigenic determinant II) of NDV's F protein (aa 65–81; Toyoda *et al.* 1988) or an eight aa neutralizing epitope of the NDV HN protein (aa 346–353; Chambers *et al.* 1988) in the βH-βI loop of the CMV CP. While technically a subunit vaccine, presentation of the epitope on the surface of a plant virus particle results in a regular array that has the capacity to stimulate the immune system to a higher extent than a soluble subunit. Recombinant CMV CP was expressed from infectious cDNA constructs of the Ixora strain of CMV. *In vitro* RNA transcripts were generated and used to inoculate *Nicotiana benthamiana*.

We report here that when the HN epitope was engineered into the internal, surface-exposed motif, the modified virus was infectious and the NDV HN epitope was recognized by anti-NDV sera. The chimeric CMV CP has the potential for development into a candidate vaccine for vaccination of chicks against NDV.

Materials and methods

Recombinant plasmids

Three CMV–CP recombinants were made by addition of the F, HN, or a tandem duplication of HN (HN2) epitopes, into a surface-immunodominant region of the CMV CP. The coding sequences corresponding to the antigenic sites of the HN glycoprotein and F protein of NDV were generated from the amino acid sequences of the linear epitopes by reverse translation. Two pairs of complementary and overlapping oligonucleotide primers, HN1F/R and HN2F/R, were designed to synthesize the gene fragments that code for a monomer version (HN), and a dimer version (HN2) of the HN epitope, respectively. The nucleotide sequences of the primers, together with the respective amino acid sequences of the linear epitopes, are illustrated in Figure 1a and b. Several silent mutations were included in the primer pair HN2F/R to minimize the direct duplication of the nucleic acid sequence while encoding two identical epitopes in tandem. For synthesis of the F epitope-encoding fragment, a DNA template from previous work (pET28/NDVF/BYMV-CP) and a primer pair F1F/R was used (Figure 1c). All primer pairs were tagged with appropriate restriction sites as shown in Figure 1 for cloning. A 30-cycle polymerase chain reaction was employed to synthesize the HN and F epitope-encoding fragments under the following conditions: 1 min at 94 °C for denaturation, 1 min and 30 s at 42 °C for primers or primer/template annealing, and 1 min 30 s at 72 °C for polymerization. The amplified antigen-encoding fragments were doubly digested with *Xho*I and *Hin*dIII and cloned into plasmid vector pBluescriptII Sk(+). The resulting recombinant plasmids were named as pSKHN1, pSKHN2, and pSKF, respectively.

Integration of the antigen-encoding fragments into CMV: A plasmid (pUC3m) containing a modified cDNA copy of RNA 3 of CMV Ixora strain was used as a template to amply the 3′ portion of the CMV coat protein (CP) gene and the subsequent untranslated region (3′UTR) using primer pair 3MCF/P2 as illustrated in Figure 2a. The amplified fragment is slightly different from the template in that a *Hin*dIII site was introduced, replacing the original *Xho*I site. The amplified partial CP gene and 3′UTR (PCPUTR) was then doubly

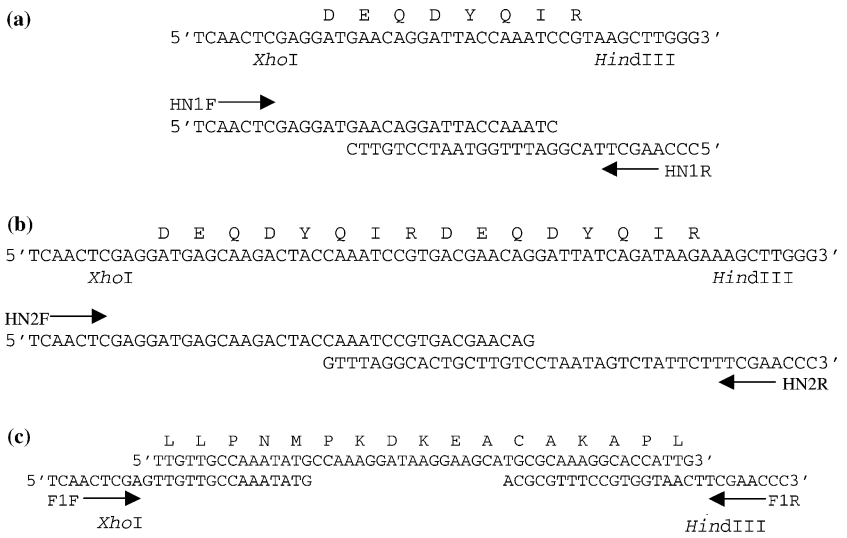

Fig. 1. Synthesis of HN (a) , HN2 (b) , and F (c) NDV epitopes using overlapping oligonucleotide primers and PCR. Restriction sites *Xho*I and *Hin*dIII were incorporated into the primers to enable cloning of fragments into the βH-βI loop in the CMV CP. Arrows indicate the 5′ and 3′ orientation of the primers. Amino acids encoded by the corresponding nucleotide sequence for the epitopes are shown above the nucleotides.

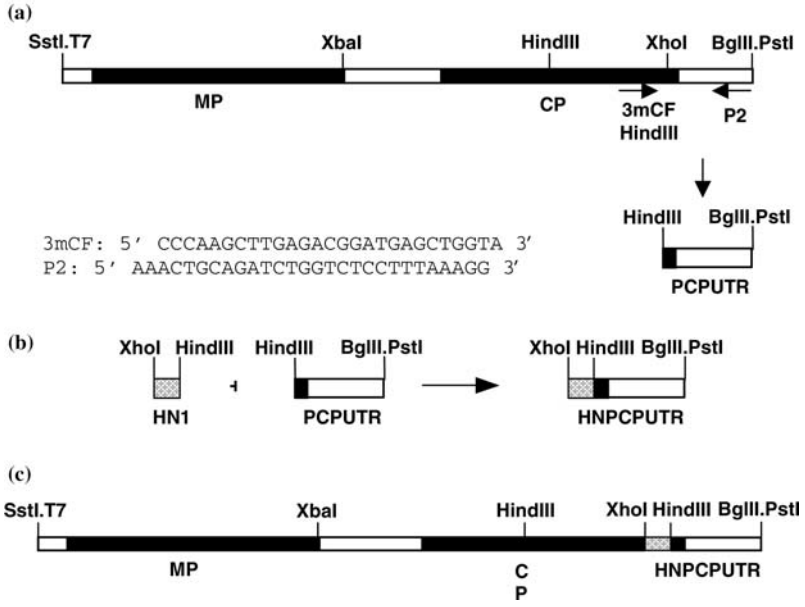

Fig. 2. Schematic diagram of the cDNA of CMV Ixora RNA3 showing integration of the HN (a) and F (b) epitopes amplified as shown in Figure 1 into the CP. Bold lines indicate the coding regions for the movement protein (MP) and coat protein (CP). Open boxes indicate the 5′NTR, intergenic region, and 3′NTR, respectively. The shaded box indicates location of the foreign epitope sequence.

digested with *Hin*dIII and *Pst*I and inserted into the eptitope-bearing plasmids pSKHN1, pSKHN2 and pSKF, respectively, generating pSKHN1/PCPUTR, pSKHN2/PCPUTR and pSKF/PCPUTR (Figure 2b). The antigen-encoding fragments were finally integrated into the RNA3 cDNA by replacing the XhoI/PstI fragment of pUC3m with the *Xho*I/*Pst*I fragment from pSKHN1/PCPUTR, pSKHN2/PCPUTR, or pSKF/PCPUTR, respectively (Figure 2c). The final constructs were named

pUC3mHN1, pUC3mHN2, and pUC3mF, respectively.These recombinants will be referred to as F, HN, or HN2, respectively.

In vitro *transcription, plant inoculation and disease symptoms*

To make infectious CMV transcripts, pNOT1 and pNOT2 (cDNA clones for CMV RNA1 and RNA2, respectively) were linearized at the *Bam*HI site and the antigen-bearing clone of RNA3 (HN, HN2, or F) was linearized at the *Bgl*II site. One μg of each linearized template was used in a separate 20-μl reaction for synthesis of capped RNA transcripts using the mMessage mMachine T7 transcription kit from Ambion Inc. (Austin, TX). The transcripts were diluted to 0.5 μg μl^{-1} with 50 mM potassium phosphate (pH 7) and a mixture of the freshly transcribed RNA1, RNA2, and the engineered RNA3 were co-inoculated on to leaves of young *N. benthamiana* plants at the 5–6 leaf stage. Three leaves were inoculated for each plant when the largest leaf was approx. 5 cm long. The inoculated plants were grown in greenhouse with a 16–8 h photoperiod at 28 °C.

Virus propagation

Twenty-eight days after the initial transcript inoculation, leaf tissues inoculated with F and HN were ground in a mortar and pestle with a phosphate buffer (pH 7.5). The virus particle-containing leaf extract were rubbed on the leaves of young *N. benthamiana and N. tabacum* plants with a gloved finger.

Reverse transcription-PCR and sequence analysis

Two methods were used for RT-PCR. Total nucleic acid was extracted using TRI Reagent (Molecular Research Center, Inc. Cincinnati, OH) from upper leaves of the inoculated plants at 2-week intervals after inoculation. Five μg total RNA was reverse transcribed in a 25-μl reaction mixture containing 20 pmol of reverse primer (P2) and 400 units of M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA). Two μl of the resultant first strand cDNA was amplified using 50 pmol each of the construct-specific forward primers (HN1F, HN2F, or F1F) and universal reverse primer P2 in a 100-μl PCR reaction mixture with 2.5 units of AmpliTaq DNA polymerase (Perkin Elmer Biosystems, Foster City, CA). The PCR fragments were fractionated on a 0.8% agarose gel.

For sequence analysis, aliquots of total RNA were amplified by conventional reverse transcription-polymerase chain reaction (RT-PCR) assays with the corresponding primer pairs (LN1, homologous to the CP 5′ terminus–ATG in bold : 5′ GTT**ATG**GACAAATCTGAATCAGC; LN36, complementary to the 3′ NTR: 5′ TGGTCTCC TTTAAAGGACCC) and utilizing the Titan RT-PCR One Tube kit (Roche Molecular Biochemicals, Chicago, IL). Sequence analysis of PCR products was performed on an ABI-PRIZM 373A Genetic Analyzer at the DNA Sequencing Facility, Center for Biosystems Research, College Park, MD. Sequence data were analyzed using Lasergene software by DNASTAR (Madison, WI). Protein structures were determined using the SWISSPROT and DeepView/Swiss-Pdb viewer V. 3.7 software to visualize the structures.

Protein extraction and Western blot analysis

CMV particles were extracted from infected plants by the method of Lot *et al.* (1972) with modifications. Briefly, the leaf tissues were ground in a blender with two volumes of 0.5 M sodium citrate (pH 6.5) and two volumes of chloroform. The crude virus particles were precipitated from the supernatant by adding PEG to give 10% (w/v). The pellets were re-suspended in a 0.05 M sodium citrate buffer and the virus particles were recovered from the suspension by centrifugation in a Beckman Type 40 rotor for 2.5 h at 39 000 rpm. Ten μg of the viral proteins, as determined by Bradford assay, were separated on a 12% SDS-PAGE and transferred to an Immobilon-P membrane (Millipore, Bedford, MA). The transferred proteins were probed with either a 1:5000 dilution of anti-CMV rabbit polyclonal anti-serum (American Type Culture Collection, Manassas, VA) followed by a 1:1500 dilution of goat anti-rabbit alkaline phosphate(AP)-conjugated serum (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD), or a 1:200 dilution of chicken anti-NDV anti-serum (Charles River Spafas, Inc. Wilmington, MA; Ser. no. 981230) followed by a 1:1500 dilution of AP-conjugated goat anti-chicken antibodies (Kirkegaard & Perry Laboratories, Inc.). Membranes were developed by the addition of the AP substrates BCIP and NBT.

Results

We constructed three hybrid viruses in which the F(LLPNMPKDKEACAKAPL) or HN (DEQDY QIR) and duplicated HN (HN2;DEQDYQIRDE QDYQIR) epitope were engineered, using PCR and complementary primers, between amino acid E at position 195 and T at 196 in the internal conserved βH-βI loop motif within the CMV CP (Figure 1). The engineered CP was then integrated into full-length RNA3 using restriction digestion as shown in Figure 2. The epitopes were predicted to form a loop in the βH-βI motif on the exterior surface of the CP (Figure 3). Upper leaves of the engineered CMV-inoculated plants were tested for systemic virus infection by RT-PCR in 2-week intervals after the initial transcript co-inoculation. The first test showed no sign of CMV infection in sample leaves, indicating the establishment and/or the movement of the engineered CMV in the host was slow (data not shown).

Twenty-eight days post-inoculation, the HN monomer-bearing CMV-inoculated plants developed mild viral symptoms (Figure 4); the F epitope-bearing CMV showed more severe symptoms, but still less severe than that of WT CMV (not shown). Both symptoms were delayed compared to WT CMV. RT-PCR analysis using primers flanking the CMV CP resulted in the expected HN and F bands (Figure 5). Sequence analysis of RT-PCR products amplified from total nucleic acid extracts of individual plants showed that deletions of one to several amino acids of the HN and F epitope can occur (Table 1). The HN2 construct was not infectious.

To test the stability of the F and HN-bearing CMV, systemically infected leaves of the *N. benthamiana* host plants were used as inoculum

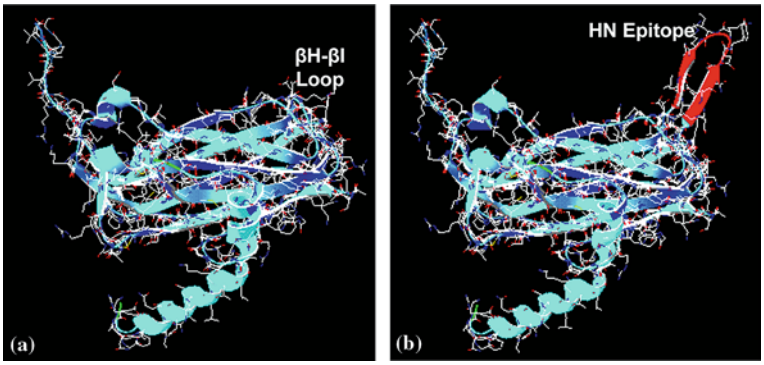

Fig. 3. Predicted secondary structure of the CMV Ixora CP without (a) and with (b) addition of the HN epitope in motif βH-βI.

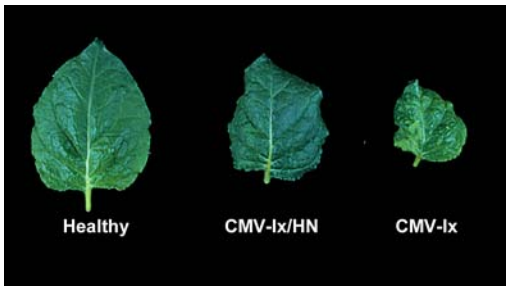

Fig. 4. Symptom expression of CMV wild type (WT) Ixora and recombinant CMV's on *N. benthamiana* leaves 28 days post-inoculation. Individual leaves of taken from plants inoculated with HN and WT show the milder symptoms of the HN construct.

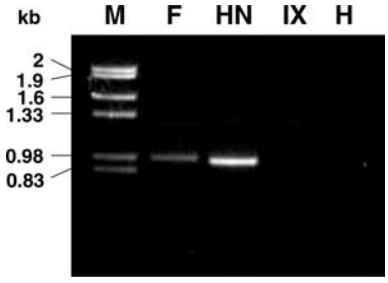

Fig. 5. Results of RT-PCR analysis of plants infected with F (F) and HN (HN) constructs using primers flanking the engineered site. M, Lambda *Eco*RI + *Hin*dIII DNA marker. WT CMV (IX) and mock-inoculated (H) leaf samples.

Table 1. Summary of Progeny Analysis of F and HN fusions in the βH-β1 loop.

Construct	No. of sequenced RT-PCR products	Amino acid sequence of the insert (bold: aa's of the βH-β1 loop epitope flanking the insertion site)		
		DDALE	———————	**TDE**
F	10	9/10	**LLPNMPKDKEACAKADLKLE**	
		1/10	LLPNMPKDK–ACAKAPLKLE	
HN	6	4/6	DEQDYQIRKLE	
		2/6	DEQ- - - - - - - -	

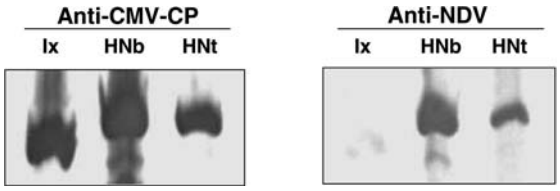

Fig. 6. Expression of HN antigen from CMV-Ix/HN as analyzed by immunoblotting. Ix, HNb (*N. benthamiana)*, HNt (*N. tabacum*) using antibodies to CMV or NDV.

to multiply the engineered viruses in both *N. benthamiana* and *N. tabacum* plants for immunological studies. Eighteen days after the re-inoculation, RT-PCR was performed on the leaf samples of the host plants to confirm the systemic infection and the presence of the HN antigen-encoding sequence in the virus progenies. The F epitope was not stable (data not shown). The coat protein from the progeny virus from HN was tested for its reactivity to anti-NDV anti-serum produced by an NDV-challenged chicken. Figure 6 shows that the coat protein of the HN epitope-bearing CMV was reactive with the chicken anti-NDV anti-serum in both *N. benthamiana* and *N. tabacum*. A replica membrane was tested with rabbit anti-serum raised against CMV as a control. From either 3.5 gm of *N. benthamiana* leaves or 7 gm of *N. tabacum* leaves, approx. 1.2–1.5 mg of purified HN-bearing CMV virus particles were obtained.

Discussion

Expression of immunogenic peptides as fusions with surface loops of plant virus capsid proteins has been used for the generation of numerous candidate animal vaccines (Pogue *et al.* 2002; Pogue & Lomonossoff, 1998). These plant-produced proteins can be delivered as oral vaccines in dried or partially processed leaf tissue (Streatfield & Howard 2003). This strategy provides an opportunity for development of an oral poultry vaccine for large-scale vaccination of chicks against NDV. In this study, two peptides, one from the F protein and one from the HN protein of NDV, were fused to the CMV CP to examine the utility of the CP in serving as a carrier protein for these neutralizing epitopes.

CMV is a wide host range virus that accumulates to significant levels in infected leaves of host plants, depending on the strain of CMV and the host (Palukaitis *et al.* 1992), and its CP has been shown to accommodate a foreign peptide fusion of 27 aa (Natilla *et al.* 2004). As the atomic structure of CMV is known, one can design fusion proteins in which foreign peptides are exposed on the surface of the virion (Smith *et al.* 2000). Because the capsid protein is multifunctional, playing a role in encapsidation, insect transmission, virus movement, symptom expression and host range, modifications to specific protein domains may disrupt one or more of these functions. We chose to introduce peptide epitopes into the conserved, prominent βH-βI surface loop. We also examined the effect of introducing the F epitope in the *N*-terminus of the CP, which contains a high concentration of basic amino acids arranged in a helix and is involved in protein-RNA interactions, and the *C*-terminus of the CP forms extensive interactions between pentameric and hexameric structures of the virion (Smith *et al.* 2000).

We found that deletions of amino acids in the introduced epitopes occurred in individual

plants, and that duplication of the HN epitope rendered the virus non-infectious. The F epitope insertion was not as stable in the passaged virus as the HN epitope. Why and how are these internal deletions occurring? The pI and/or size of the epitope may affect stability of the virion, as shown in *Cowpea mosaic virus* (CPMV), another isometric virus (Porta *et al.* 2003) and *Tobacco mosaic virus* (TMV), a rod-shaped virus (Bendahmane *et al.* 1999). The βH-βI loop is negatively charged and contains five strongly acidic residues (pI 3.04 with a −5.08 charge at pH 7), and mutations in the loop that reduce the negative charge affect insect transmissibility, but do not apparently affect virion assembly (Liu *et al.* 2002); however, stability of virions may be affected (Ng *et al.* 2000). Although addition of the F and HN epitopes does not significantly affect the overall pI of the coat protein, local changes in the loop may occur as the pI of the F epitope is 8.1, the pI of HN is 4.1, and the pI of HN2 is 3.9. Natilla *et al.* (2004) stably expressed a 27 aa peptide epitope with a pI of 10.04 in this loop, therefore, instability of the HN epitope, and non-infectivity of the HN2 construct cannot be explained solely based on size of the insertion or charge of the epitope. Instability of epitopes in CPMV were found to occur by proteolytic cleavage (Taylor *et al.* 1999), or amino acid changes that collectively reduced the pI of the inserted peptide (Porta *et al.* 2003). We did not observe either. Folding predictions using SWISSPROT were only able to be generated for the HN epitope insertion into the CP which is represented in Figure 3. Folding predictions of the the F and HN2 epitope insertions generated truncated CP molecules (not shown). Therefore, instability may be due to improper folding of the CP and loss of epitope due to reduced fitness.

Recombinant CMV containing the engineered HN epitope in the internal motif was, for the most part, stably infectious in initially infected plants and after passaging; anti-sera to NDV was able to recognize the HN epitope on the purified virus particles (Figure 6). Immunogenicity of the HN epitope suggests that it adopts a conformation in the CMV CP environment similar to its native conformation, and may result in generation of neutralizing and protective antibodies when used as a vaccine.

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