RESEARCH REPORT
SWINE HEALTH

Title: Development of Edible Vaccines against PRRSV: A Proof of Concept Study – **NPB #06-128**

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II. Industrial Summary

The overall objective of this project is to develop edible vaccines to combat the devastating porcine reproductive and respiratory syndrome (PRRS) as an alternative to current MLVs. The goal is to provide pork producers with cheap, effective, and safe vaccines against PRRSV. Corn, as the major feedstock of pigs, was studied to express the immunogenic PRRSV proteins. To expedite the study, we first used maize callus (cultured maize cells) to express the proteins of interest. The expressed protein was identified, and the protein's immunogenicity was studied in mouse model. Our results indicated that the major immunogenic PRRSV protein can be successfully expressed in maize callus, and the protein interacted with antibodies developed against PRRSV. The mouse experiments, however, produced inconclusive results about the immunogenicity of the protein expressed in maize callus. The main reason is that the protein expression level in maize callus is relatively low and could not be quantified. Thus, we could not determine how much of the target protein was administered to the test animals. The lack of strong antibody response could very well be attributed to the insufficient amount of target protein administered. Nevertheless, our results have shown that maize is able to express recombinant PRRSV proteins. Although more work needs to be done to increase the protein expression level (currently being investigated) and to study the immunogenicity and antigenicity of the recombinant proteins in mice and pigs, maize expressed viral proteins clearly still hold great promise as effective vaccines against the virus. The continuation and success of this project will have a significant impact on our combat against this devastating virus and the swine producers. Not only will new and safer vaccines be produced, but the vaccines will be much cheaper and extremely suitable for mass immunization. Swine producers will be directly benefited from purchasing cheaper and easy-to-be-administered vaccines and from the improved productivity of the swine farm.

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III. Scientific Abstract:

Porcine reproductive and respiratory syndrome virus (PRRSV) has caused a pandemic that has proven extremely costly for the world pork industry. Insufficient vaccination methods are one reason the emergence of PRRSV has had such brutal consequences. Current modified live virus (MLV) vaccines have several shortcomings: they are often ineffective against varying strains of PRRSV and can revert back to pathogenic PRRSV.

The aim of this work was to investigate the possibility of developing a subunit vaccine in maize as an alternative to current MLVs against PRRSV. A maize-optimized ORF5 DNA sequence was designed for glycoprotein 5 (GP5), the major envelope protein of PRRSV. This DNA sequence was used to create a vector to transform maize callus via particle bombardment. Maize callus was transformed. Callus was cultivated, and screened for ORF5 DNA by PCR and GP5 protein by SDS-PAGE and western blot. Protein was extracted from callus, and tested in vivo in mice for antigenic response to GP5. Mouse immune response in serum was determined using ELISA

Screening revealed several lines of maize callus that produced detectable quantities of GP5. Protein extract from this callus, when administered intramuscularly, with or without adjuvant, or administered orally, however, did not produce conclusive results about the immunogenicity of the protein. The main reason is that the expression level of the recombinant protein is low. Although clearly detectable, the protein's quantity could not be affirmatively determined, and that created a problem in determining the quantity administered to the test animals. The lack of strong antibody response over that of negative controls could very well be attributed to the insufficient amount of target protein administered. Thus, investigating new transgene construct with different promoters will be of high priority to increased recombinant protein expression level in maize, since this determines whether or not transgenic maize could be ultimately used as vaccines for PRRSV.

IV. Introduction

Porcine reproductive and respiratory syndrome (PRRS) has devastated swine industry and resulted in tremendous economic losses worldwide. Currently, the control of PRRS has largely relied on modified live-attenuated vaccines (MLVs). However, the versatility and safety of MLVs are of major concerns as MLVs such as Ingelvac® PRRS MLV have been shown to revert to a pathogenic phenotype and causes diseases in pigs. On the other hand, edible plant vaccines have been shown to elicit mucosal and systemic immune responses and confer protection. Corn is selected as the recombinant protein expression host because it is the primary feedstock for swine and previous research has found that corn grain provides natural protection, "bioencapsulation", for recombinant proteins to defer gastroenteric degradations during immunogen delivery. The goal of this project is to develop transgenic corn expressing PRRSV immunogens as edible recombinant vaccines to help eliminate the PRRS viruses from swine herds. Since PRRSV ORF5 (GP5) and ORF6 (M) proteins have been shown to be immunogenic, we will first study the feasibility of expressing recombinant GP5 and M proteins in transgenic maize. The expression levels of these proteins will be determined and the immunogenicity of the proteins will be tested in mice and pigs. Then the expression level of the recombinant proteins will be optimized by using different transgene constructs. Finally, the efficacy of the recombinant subunit vaccines will be evaluated. Successful completion of this project will provide the swine industry a new, safer, and cheaper vaccine against PRRSV to effectively prevent and control this economically important swine disease.

V. Objectives

- Obj. 1: Feasibility study of expressing recombinant GP5 in transgenic corn (callus)
- Obj. 2: Generation of purified GP5 and M proteins from PRRSV obtained from cell culture
- Obj. 3: Establishment of transgenic maize expressing PRRSV ORF5 and ORF6 gene individually and combined and characterization of the recombinant proteins
- Obj. 4: Determination of the immunogenicity of recombinant GP5 and M protein (alone or in combination) in pigs by oral and I.M. routes of immunization

VI. Materials and Methods

The cDNA of ORF5 was produced from PRRS virus (strain: ATCC VR 2385) using reverse-transcriptase PCR. The cDNA of ORF5 was optimized based on the codon usage preferences of maize by Genscript Corporation (Piscataway, NJ) cloned into a pUC-57 plasmid at the Kpn I and Sac I restriction sites on the 5' and 3' ends of the cDNA, respectively. One plant expression vector (pORF5) was made with the ORF5 gene downstream of a dual-enhanced Cauliflower Mosaic Virus 35S promoter (CaMV35Sde) and a TEV leader. After PCR, DNA digestion and DNA sequencing were carried out to confirm the authenticity of the ORF5 insertion.

Biolistic-mediated transformation was performed at Iowa State University to create transgenic maize calluses. The pORF5m construct was co-bombarded with selectable marker plasmid containing BAR gene, which confers resistance to bialaphos. Calluses were passed through a series of media for proper selection and growth. Selected calluses were maintained on N6S media [4 g/L N6 salts, 1 ml/L N6 vitamin stock, 2 mg/L 2,4-D, 100 mg/L myo-inositol, 30 g/L sucrose, 2.5 g/L gelrite, pH 5.8] on Petri plates, in dark growth chamber at 28 ºC.

For protein extraction, maize callus was ground to fine powder under liquid nitrogen. Extraction buffer [200mM Tris–HCl, (pH 8.0), 100mM NaCl, 400mM sucrose, 10mM EDTA, 14mM 2-mercaptoethanol, 0.05% Tween-20] was added at a ratio of 1 μL:1 mg was added to callus powder and placed on ice for 10 min. Samples were centrifuged for 10 minutes at 15,000 G. Supernatant was removed and the centrifugation procedure was repeated 1-2 more times to remove remaining callus particles.

Samples were run on NuPage 4-12% Bis-Tris gels (Invitrogen, Carlsbad, CA). Protein was transferred to Immun-Blot PVDF membrane (0.2 μm) from Bio-Rad (Hercules, CA). Western blot was performed using Immun Star HRP substrate kit from Bio-Rad. The primary antibodies used were polyclonal (mono-specific) antibodies (rabbit) against PRRSV GP5 (kindly provided by Dr. Serge Dea and Dr. Carl Gagnon), and the secondary antibodies used were goat anti-rabbit conjugated with horse radish peroxidase. Bands were detected via chemilumine-scence. All chemicals are purchased from either Sigma (St. Louis, MO) or Fisher (Itasca, IL).

To generate purified GP5 from PRRSV, the following procedures were used:

1) PRRSV (VR-2385) was propagated in the highly permissive MARC-145 cell line.
2) The cell culture was harvested, and the host cells were lysed by consecutive freeze/thaw cycles. After removing cell debris, virion particles were concentrated by ultracentrifugation through sucrose cushion.
3) Crude purified virions were solubilized by a buffer containing a detergent, and the proteins in the supernatant were separated by a cation exchange chromatography process followed by a hydrophobic interaction chromatography.

The immunogenicity study for recombinant GP5 were conducted by oral and I.M. routes of immunization in mice with proper negative and positive controls. Details are shown in the result section.

VII. Results

<u>Objective 1: Feasibility study of expressing recombinant GP5 in transgenic corn (callus)</u>

The plant expression vector (pORF5) construct is shown in Figure 1. The vector was delivered into corn calluses by biolistic bombardment. The calluses were co-bombarded with a plasmid containing the bar gene that confers bialaphos resistance at Iowa State University. Transgenic corn calluses were shipped to Virginia Tech in Petri dishes and propagated in N6 selection medium (N6S) (Figure 2). The protein extract from described transgenic maize calluses were obtained by the method above, and the SDS-PAGE of the protein samples is shown in Figure 3. A unique protein band is evident in the transgenic callus extracts, which has an apparent molecular mass of ~28 kDa. This band is clearly absent in the protein extracts obtained from non-transgenic control maize calluses (lane 2 and 7). Western blot with mono-specific anti-GP5 antibodies was done to determine the antigenicity of the unique protein. The Western blot of maize callus protein extracts is shown in Figure 4. The unique band in Figure 3 is shown to interact with the mono-specific antibodies against PRRSV GP5.

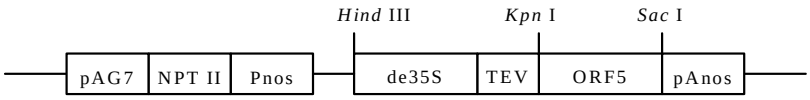

Figure 1. Construct map of the plant expression vector. pAg7: agropine synthase polyadenylation sequence; NPT II: neomycin phosphotransferase II gene (kanamycin resistance); Pnos: nopaline synthase promoter; de35S: double enhanced cauliflower mosaic virus 35S promoter; TEV: tobacco etch virus translational enhancer; pAnos: polyadenylation sequence of the nopaline synthase terminator.

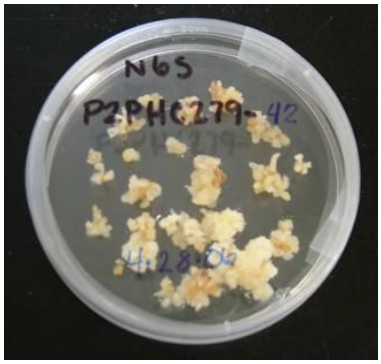

Figure 2. Transgenic corn callus maintained on N6S medium.

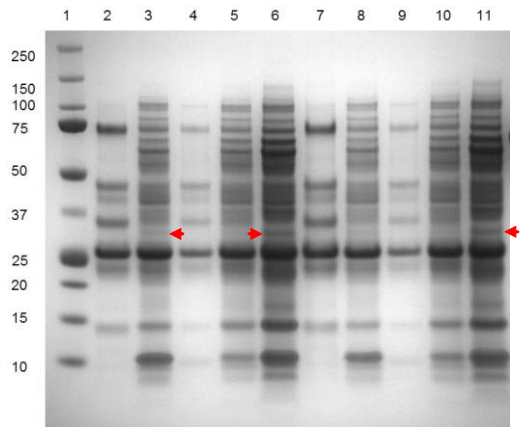

Figure 3. SDS-PAGE of protein extracts from maize calluses. Lane assignments: 1, MW marker; 3-6, 8-11, transgenic callus extracts; 2&7, non-transgenic callus extracts. Coomassie blue stained.

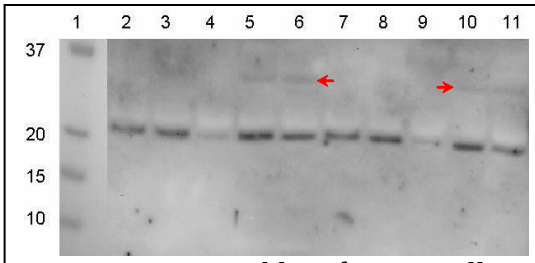

Figure 4. Western blot of maize callus protein extracts. Lanes 1-11 are identical to those in Figure 3. The red arrows point to the GP5 band. The molecular marker at 25 kDa does not show up well.

Objective 2: Generation of purified GP5 and M proteins from PRRSV obtained from cell culture

First of all, since the antibodies available to us could not identify the presence of M protein in lysed viral particle, thus our work has been concentrated on developing a process to purify GP5 from cell culture.

PRRSV was first propagated in MARC-145 cells. PRRSV virions were first purified and concentrated through sucrose cushion ultracentrifugation. GP5 protein was subsequently solubilized with Triton X-100 detergent for further processing. Cation exchange chromatography (CEX) was utilized for partial fractionation of GP5, although the viral nucleocapsid protein (N) was a major impurity in CEX elution fractions. Figure 1

shows a silver stained reduced gel and western blot of cation exchange chromatography fractions. The arrows point to GP5 and N protein, respectively.

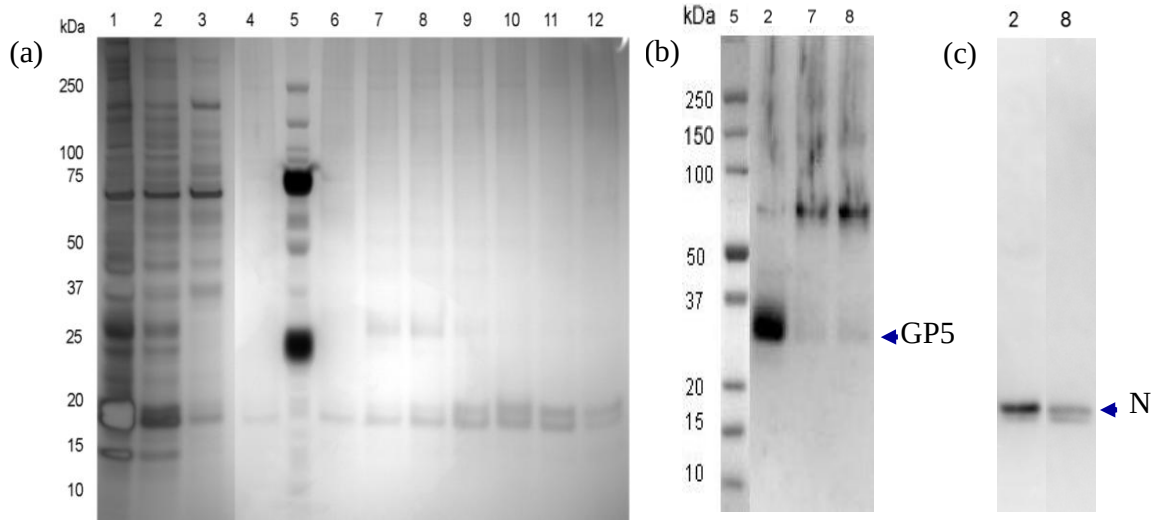

Figure 5. (a) Silver stained SDS-PAGE gel and western blots detected by (b) mono-specific anti-GP5 (αGP5) and (c) polyclonal antiserum (αPRRSV) of CEX fractions recovered during linear gradient elution. L1: PRRS virions. L2: PRRS virions solubilized with Triton X-100 after NaCl removal. L3: pooled/concentrated flow through fractions. L5: protein marker. L4-L12: elution fractions from cation exchange chromatography.

As shown in Figure 1, GP5 appears as a diffuse band at 25-27 kDa (Fig. 5 b), which probably indicates the heterogeneous glycosylation patterns on GP5. A distinct band also appears at ~ 60 kDa, which could indicate the presence of a GP5 dimer. GP5 was further purified by a second chromatographic step, hydrophobic interaction chromatography (HIC) by means of a two-stage elution scheme. Pure GP5 protein was eluted from the HIC resin in the second HIC elution stage by Triton X-100 displacement; however the protein is present as a homodimeric/tetrameric aggregate as shown in Figure 6 (lanes 10 and 11 in Fig. 6 b).

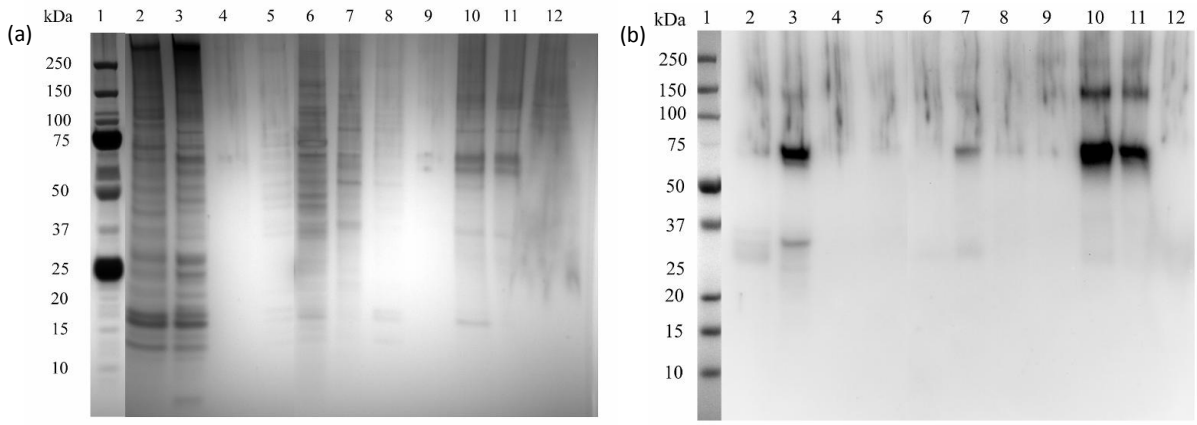

Figure 6. (a) Silver stained SDS-PAGE gel and (b) western blot (αGP5) of HIC fractions. L1: protein marker, L2: fraction loaded, L3-12: fractions collected during HIC.

However, the above process is not scalable in order to produce larger quantities of the viral proteins. Thus part of our current effort has been devoted to develop a scalable process to generate purified PRRS virions. The following outline the procedures we are using to develop virion purification process:

1) PRRSV (VR-2385) was propagated in the highly permissive MARC-145 cell line.

2) The virions were released by three consecutive freeze/thaw cycles of the infected cells. After removing cell debris, virion particles were concentrated by ultrafiltration with a polyethersulfone-membrane which has a molecular weight cut-off of 300 kDa.
3) The virus particles in ultrafiltration retentate were separated by heparin affinity chromatography.

Figure 7 shows the elution of PRRS virion during heparin affinity chromatography after ultrafiltration of cell lysis. Figure 8 shows the gel electrophoresis analysis of the samples at different stage of virus purification. Evidently, PRRSV particles have been purified to certain extent (Lane 4 in Fig. 8, the sample was collected during the elution of the sharp peak in Fig. 7) as indicated by the presence of GP5 and N proteins in the fraction. However, there is clearly a need for further purification to generate pure virus particles because the presence of other impurities.

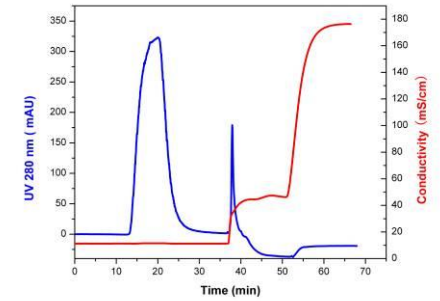

Figure 7. Heparin affinity chromatography of 3.5 ml ultra-filtration retentate.

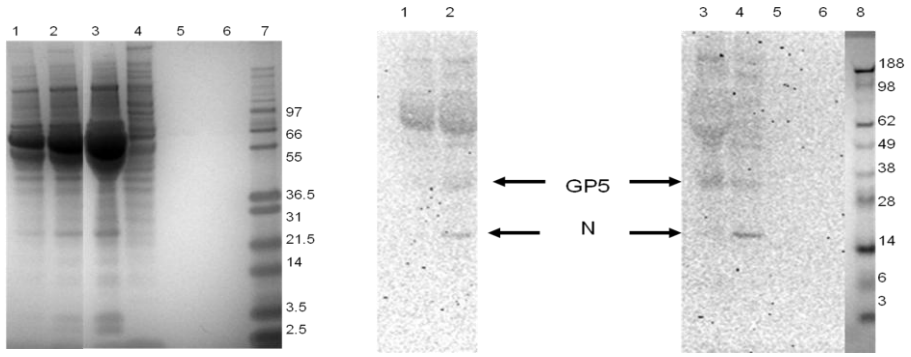

Figure 8. (a) Commassie stained SDS-PAGE gel and (b) Western blot of purification process using polyclonal anti-serum as primary antibody and protein-G HRP conjugate as secondary antibody. L1: clarified sample; L2: ultrafiltration retentate; L3: 10×concentrated flowthrough (Fig.3, the large peak); L4: 10×concentrated elution peak; L5: 10×concentrated fraction at 2 M NaCl in Tris-HCl buffer elution; L6: 10×concentrated column regeneration fraction; L7: Unstained standard; L8: Prestained standard.

<u>Objectives 3: Establishment of transgenic maize expressing PRRSV ORF5 and ORF6 gene individually and combined and characterization of the recombinant proteins</u>

The presence of recombinant GP5 in transgenic maize callus is confirmed by western blot analysis as shown in Figure 9. Exposure to anti-GP5 antibody resulted in a faint band at 30 kDa in nine lines of callus samples. A similar, but a much darker band, occurred in lanes containing protein purified from PRRSV (Lane 3). This contrast indicated that GP5 was present, but in low concentrations or in a form that had lower affinity for the primary antibody. Meanwhile, another band occurring at approximately 60 kDa may have been a dimer of GP5. This band occurred for both samples from callus and for protein purified from PRRSV. The potential dimer was more obvious in callus samples than PRRSV protein, which could indicate that expression, modification, or storage differences that cause the dimer to be more prevalent in callus samples. Nevertheless, it is evident that corn is a feasible host to express recombinant GP5.

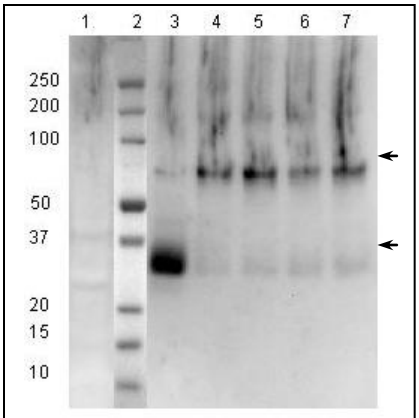

Figure 9. Western blot of transgenic maize callus (L4-7), non-transgenic maize callus (L1), and PRRSV reacting with monospecific anti-GP5 antibody. L2: molecular marker. L3: PRRSV virus protein.

On the other hand, we are working on generating ORF6 gene construct for maize transformation. To ensure relative high-level expression of ORF6, we designed the sequence for synthesis. Briefly, maize codon preference was used for the coding sequence of ORF6. Meanwhile, such DNA sequences as the potential polyadenylation signal and ATTT sequence which may cause mRNA selective degradation were removed. Sequences around the translation initiation site and terminal site were also changed to conform to the eukaryotic consensus sequence. The designed sequence was synthesized by Genscript (Piscataway, NJ, USA). Now we are constructing the expression vector by replacing *gus* gene in pAHC25 (Christensen and Quail, 1996) with a 580 bp *Xma*I-*Sac*I fragment harboring ORF6. ORF6 will be under the control of a strong monocot promoter, maize ubiquitin promoter.

Objective 4: Determination of the immunogenicity of recombinant GP5 and M in mice by oral and I.M. routes of immunization

The immunogenicity of recombinant GP5 expressed in transgenic maize callus was tested in mice. In total, 48 mice were divided into 6 groups for immunogenicity study of recombinant GP5. The specific studies are outlined in Table 1. All animal studies were carried out in the Central Vivarium animal facility at Virginia Tech.

Table 1. Immunogenicity study of recombinant GP5 in BALB/c mice.

Group number	Number of mice	Vaccine	Route of immunization
1	8	PBS buffer	Intramuscularly (I.M.)
2	8	recombinant GP5,	I.M.
3	8	rGP5 with Freund's incomplete adjuvant	I.M.
4	8	Transgenic corn callus	Oral
5	8	Corn callus with Freund's incomplete adjuvant	Oral
6	8	GP5 from *E. coli*	I.M.

All mice received the same dose of the vaccine received by their study group and a booster two weeks after the initial inoculation. Blood was drawn prior to the initial inoculation, just before the booster inoculation, and every two weeks after the booster. The collected sera samples were tested by ELISA. Figure 6 shows the ELISA analysis results.

As indicated in Fig. 10, average immune response was highest for recombinant GP5 purified from *E coli*. Immune response increased greatly in the first two weeks after administration, then remained stable over the course of the study. Immune response was significantly less for other test groups. Groups with protein extracted from transgenic callus, administered orally or intramuscularly resulted in similar immune response to the test group with PBS. The group given GP5 from maize extract with adjuvant exhibited greater immune response after 40 d than those without adjuvant. However, groups given protein extract from non-transformed maize performed similarly.

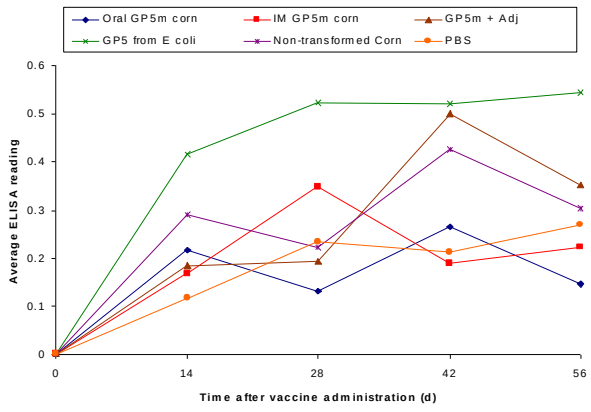

Figure 10. Average ELISA absorbance over time of serum from mice administered plant protein extract containing GP5. Groups include: GP5 from maize callus administered orally and intramuscularly (IM), GP5 from maize callus administered intramuscularly with Freund's adjuvant, GP5 purified from E coli, non-transformed maize callus, and phosphate buffered saline (PBS).

It is worth to point out that ELISA plates showed significant plate to plate variation, and the mere evidence that mice injected with PBS produced similar response indicates that these ELISA results are not

reliable. This may be explained by that the anti-GP5 antibodies are not specific, and there are some non-specific bindings from mouse sera that significantly interfere with the analysis. This problem may be solved by increasing the recombinant protein expression levels in transgenic maize callus or better prepared antibodies.

VIII. Discussion

The unique band in Figure 3 and Figure 9 has an apparent molecular mass of ~28 kDa, which is the predicted molecular mass of glycosylated GP5 (the molecular mass of un-glycosylated GP5 is ~23 kDa). In addition, this band migrated to relatively the same position on the gel as the GP5 derived from PRRSV obtained from cell culture (Figure 9). Combining with the fact that the protein interacts with the mono-specific antibodies against PRRSV GP5 in Western blot analysis (Fig. 9), it is concluded that corn is a suitable host for expressing recombinant GP5 as a potential vaccine candidate for PRRSV.

In addition to the reasons stated above for the inconclusive ELISA test, another reason is the low expression level of recombinant GP5 in maize callus. Although the protein can be unambiguously detected, the unarguable fact is that the quantity of the protein could not be determined analytically. Therefore, we did not know the exact amount of protein administered to the mice. The low antibody response could very well be attributed to the low recombinant protein administered. Future work thus needs to focus on optimizing the expression level of recombinant GP5 in maize callus.

On the other hand, developing a scalable process to purify native PRRS virions could have a significant impact to the vaccine development for PRRSV. If successful, not only will we be able to purify relatively large quantities of PRRS virus and subsequently its proteins for the development of the badly needed antibodies, but also we could use the purified viral proteins in animal tests to assess the efficacy of subunit vaccines expressed in other protein expression hosts.

IX. Publications

Matanin, B., Meng, X.J., and Zhang, C. 2008. Purification of the major envelop protein GP5 of porcine reproductive and respiratory syndrome virus (PRRSV) from native virions. *Journal of Virological Methods*. **147**:127-135.

Hu, J., Meng, X.J., and Zhang, C. Development of a scalable process for the purification of native PRRS virions from cell culture. In preparation.

Presentations:

Matanin, B., Meng, XJ, Halbur, PG, and Zhang, C. 2007, Purification of the major envelop protein GP5 of PRRSV from native virions. 2007 International porcine reproductive and respiratory syndrome (PRRS) symposium. Nov. 30 – Dec. 1, Chicago, IL.

Hu, J., Meng, X.J., and Zhang, C. 2008. Purification of native PRRS virions from cell culture. 2008 International porcine reproductive and respiratory syndrome (PRRS) symposium. Dec. 5 – 6, Chicago, IL.

X. References

Christensen, A.H. and Quail, P.H. (1996) Ubiquitin promoter-based vectors for high-level expression of selectable and/or screenable marker genes in monocotyledonous plants. *Transgenic Res.* **5**: 213-218.