RESEARCH REPORT
SWINE HEALTH

Title: Development of Edible Vaccines against PRRSV: A Proof of Concept Study – **NPB #04-171**

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Abstract

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 be able to elicit mucosal and systemic immune responses and confer protection. The long-term goal of this project is to develop transgenic corns expressing PRRSV immunogens as edible recombinant vaccines to help eliminate the PRRS virus from swine herds. PRRSV ORF5 (GP5) and ORF6 (M) proteins will be expressed in transgenic corn as recombinant vaccines since they have been shown to induce neutralizing antibodies. Corn is selected as the recombinant protein expression host because it is the primary feedstock for swine and previous researches have found that corn grain provides natural protection, "bioencapsulation", for recombinant proteins to defer gastroenteric degradations during immunogen delivery. To achieve the overall goal, the feasibility of expressing recombinant GP5 and M protein will be evaluated in transgenic corn callus followed by optimizing the expression levels of the proteins by using different transgene constructs; the immunogenicity of separately or co- expressed proteins will be tested in pigs, and finally, the efficacy of the recombinant subunit vaccines will be evaluated.

Introduction

Current PRRSV vaccines rely on MLVs, which are all based on a single isolate, but the outbreaks of PRRS continued to occur periodically in vaccinated herds, indicating that current MLVs can not fully protect pigs against infection with the genetically diversified field isolates of PRRSV. Furthermore, the reversion of a commercial MLV vaccine to a pathogenic phenotype in vaccinated pigs has been demonstrated (Botner et al., 1997; Oleksiewicz et al., 1998; Storgaard et al, 1999), and many vaccine-like and vaccine-derived PRRSV isolates have recently been isolated and shown to cause diseases in pigs (Opriessnig et al., 2002; Key et al., 2003). These all call for urgent development of a new generation of vaccines with enhanced safety, robustness, and economical feasibility.

GP5 and the M protein of PRRSV are selected as the potential recombinant vaccine candidates. GP5 of PRRSV has been shown to be immunogenic, and monoclonal antibodies directed against GP5 are neutralizing (Pirzadeh and Dea, 1997). In addition, the heterodimer of GP5 and M protein has been shown to induce neutralizing antibodies against Equine Arteritis Virus (EAV) (Balasuriya et al., 2000, 2002) and to elicit enhanced immunogenicity against PPRSV (Jiang et al., 2006).

Plant systems are very cost-efficient in biomass production, and they don't carry viruses and other pathogens dangerous to mammals. If the vaccine antigens are produced in the edible parts of the plants, they can be directly consumed and provide an easy way for mass immunization. In addition, plant vaccines, especially grain crops, possess numerous advantages compared to MLVs, such as: 1) multiple vaccines against genetically diversified virus isolates may be produced together, 2) reduced need for a cold chain during storage and transport, 3) reduced need of veterinary medical assistance in administration, and 4) elimination of concerns over transmitting infectious diseases through needle reuse or sharing which is common in veterinary practice. Furthermore, plant-based edible vaccines have been shown to be effective against various human and animal viruses. For example, the B-subunit of *Escherichia coli* heat-labile enterotoxin (Lt-B) and the spike protein of swine transmissible gastroenteritis virus (TGEV) expressed in corn and potato delivered orally elicited protective immune responses in mice, piglets, and human trials (Streatfield et al., 2001, 2003; Tacket et al., 2004; Tacket, 2005).

Edible vaccines can induce both mucosal and systemic immune responses. This feature is of great importance, since the primary gateway for many human and animal pathogens is the mucosal tissues of gastrointestinal, respiratory and genital tract. Vaccines that are administered parenterally can usually prevent systemic spread of invasive pathogens, but do not prevent infection of mucosal tissues. Thus it is essential to build up the first line of defense in mucosal system by orally fed vaccines. In addition, expression of antigens in plant cells, particularly in plant seeds, such as corn (Baily, 2000), provides "bioencapsulation" of the antigen enabling the antigen to survive the full repertoire of degrading conditions of gastrointestinal tract such as acidic pH, proteases, and bile acids and thus to maintain its potency to induce immune response effectively (Streatfield and Howard, 2003).

However, compared with parenteral immunization, orally delivered antigens are commonly less efficient. Even though as much as transgenic plant material can be fed, especially when the material is a natural component of the animal's diet, such as corn for pigs, it is preferred to have high expressions of the antigen in the plant to prevent prolonged immunization schedule and repeated exposure of the immunization animals to the antigen. Having transgenic corn with high expression of antigens will thus decrease the risk of developing immune tolerance in animals and the demand of plant materials. Therefore, optimization of antigen expression level in transgenic plants will be a key to the successful development of edible plant vaccines.

Objective

The overall goal of this project is to use the major swine feedstock, corn, as a recombinant vaccine carrier to develop edible recombinant vaccines to help eliminate the PRRS viruses from swine herds. The specific objective for the funded period was to study the feasibility of expressing recombinant GP5 in transgenic corn callus. The goal is to use the relatively easy to generate maize callus to study whether or not recombinant GP5 can be successfully expressed in transgenic maize.

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).

Results

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 medium (N6S) (Figure 2).

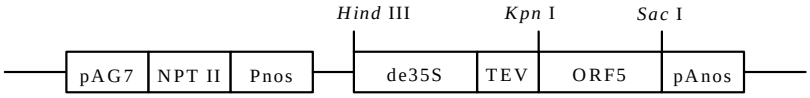

Figure 1. Construct map of the plant expression vector. pAg7: agropine synthase polyadenylation sequence; NPT II: neomycin phosphotransferase II gene (kanamycin resistance); Pnos: nopaline synthase promoter; de35S: double enhanced cauliflower mosaic virus 35S promoter; TEV: tobacco etch virus translational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

The protein extract from transgenic maize calluses were obtained by the method described 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.

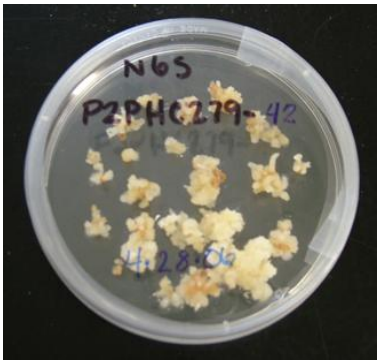

Figure 2. Transgenic corn callus maintained on N6S medium.

Discussions

The unique band in Figure 3 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 5). Combining with the fact that the protein interacts with the mono-specific antibodies against PRRSV GP5 in Western blot analysis, it is concluded that the unique protein band in Figure 3 is recombinant GP5 and thus the attainment of the project objective for the funded period. Furthermore, a sample of the protein band has been submitted for protein sequence analysis. After further confirmation of the recombinant GP5 in maize callus by amino acid sequencing, a preliminary immunogenicity study using mouse model will be carried out.

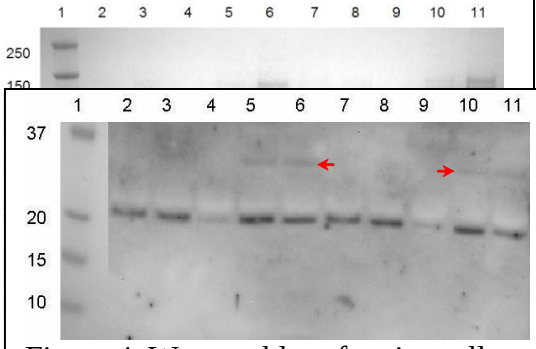

Figure 4. Western blot of maize callus protein extracts. Lanes 1-11 are identical to those in Figure 3. The red arrows point to the GP5 band. The molecular marker at 25 kDa does not show up well.

transgenic callus extracts. Coomassie blue stained.

Moreover, we have initiated the development of processes to purify GP5 and M protein from PRRSV generated from cell culture (an objective for the second stage of the project). Figure 5 shows the Western blot analysis of the resolubilized PRRSV particle (obtained from MARC-145 cell culture) in a detergent, sarkosyl. As pointed above, PRRSV GP5 migrated to relatively the identical position in the gel as the unique protein band in Figure 3. The processes to obtain purified viral GP5 and M protein will be developed. The purified GP5 and M protein will be primarily used as positive controls for the recombinant vaccine studies.

Lay Interpretation

Porcine reproductive and respiratory syndrome (PRRS) is the most devastating swine disease. This project strives to develop new and safer vaccine for the elimination of PRRSV from swine herds. Previous studies have shown the GP5 and M protein on the virus surface are immunogenic and are good candidates for developing subunit protein vaccines. Corn is the major feedstock for pigs, and it is an ideal host for vaccine development. For the reported period, we have demonstrated that recombinant GP5 can be successfully expressed in transgenic corn callus. The overall goal of this project is to develop transgenic corns expressing PRRSV immunogens as edible recombinant vaccines to help eliminate the PRRS virus from swine herds. The 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.

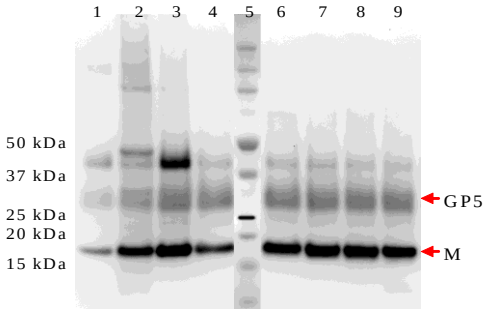

Figure 5. Western blot analysis of samples with different concentrations of detergent (sarkosyl). Lanes: 1, raw virus culture; 2, pellet after clarification; 3, pellet after sucrose cushion; 4, 6-9, viruses solubilized in 2.5%, 1.0%, 0.5%, 0.1%, and 0.05% sarkosyl, respectively; 5, molecular mass marker.

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