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Development of a Plant-based Vaccine against *Porcine reproductive and respiratory syndrome virus*: Research Progress and Future Prospects

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ABSTRACT

Porcine reproductive and respiratory syndrome virus (PRRSV) is one of a few important pathogens that threaten the pig industry worldwide. The disease caused by PRRSV is a major source of economic loss for pork producers. PRRSV infects pigs through the mucosal surface of the respiratory tract. Therefore, the production of an oral vaccine to induce specific mucosal immune response may represent the most effective approach to preventing PRRSV infection. As the production of safe subunit vaccines in systems such as mammalian, bacterial or insect cells is either impossible or too expensive, plants become a promising bioreactor. In the past several years, we have investigated the possibility of application of plants for the development of a low cost, orally administered, plant-based vaccine against PRRSV. We used the cholera toxin B subunit (CTB) of *Vibrio cholerae* as an adjuvant and the PRRSV GP5 or its neutralizing epitope (GP5-NE) as a vaccine antigen. We found GP5 or the CTB-GP5 fusion protein was no detectable in transgenic tobacco plants, though large amounts of corresponding RNA were evident. The expression level of CTB-GP5-NE in transgenic plants was in the range of 0.003 to 0.087% of total soluble proteins. The plant-derived CTB-GP5-NE was biologically active. To increase the yield of the CTB-GP5-NE recombinant protein in plant hosts, we developed a *Soybean mosaic virus*-based viral expression system. In this research review, we summarize our research progress and discuss challenges and future prospects of the development of a plant-based PRRSV vaccine.

Keywords: CTB, GP5, PRRSV, recombinant protein, viral vector

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INTRODUCTION

Porcine reproductive and respiratory syndrome is the most important infectious viral pathogen that threatens the pork industry worldwide (Albina 1997). It is caused by *Porcine reproductive and respiratory syndrome virus* (PRRSV). The disease was first found in the USA and then quickly spread widely throughout major pig-producing countries (Wensvoort *et al.* 1991; Albina 1997; Gagnon and Dea 1998; Jiang *et al.* 2008). Now, the infection has become endemic. Since its discovery, tremendous efforts have been made to control PRRSV. To date, there is no drug therapy for PRRSV infection. The virus is mainly controlled by needle injection of vaccines which include either killed/deactivated PRRSV or attenuated/modified live vaccines. However, there are several problems associated with current vaccines (Ostrowski *et al.* 2002). Dead PRRSV vaccines have proved to be poorly effective in prevention of both infection and disease (Nielsen *et al.* 1997). Modified/attenuated live vaccines generally provide at best partial protection against clinical disease but do not prevent infection (Ostrowski *et al.* 2002). Moreover, live PRRSV vaccines can revert to virulence, causing serious safety concerns (Botner *et al.* 1997; Madsen *et al.* 1998; Rowland *et al.* 1999). Therefore, a new generation of vaccines against PRRSV is urgently demanded by the pork industry.

PRRSV is a member of the artervirus family. Like other arteriviruses, PRRSV is an enveloped virus that has a single-

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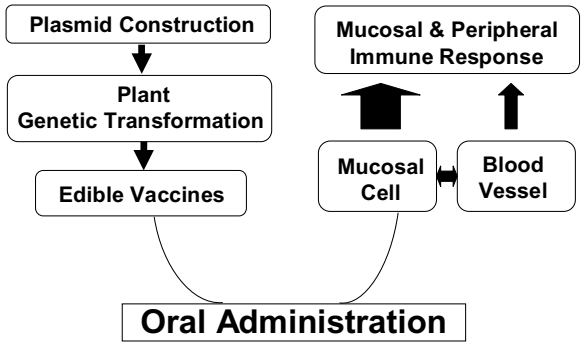

Fig. 1 The development of plant-based edible vaccines.

stranded, positive-sense RNA genome that is 14.5 kb in length. The virus produces viral proteins including the replicase, four membrane glycoproteins, i.e., GP2a, GP3, GP4 and GP5, the unglycosylated membrane protein P2b, the matrix (M) protein, and the nucleocapsid protein N in infected cells (Wensvoort *et al.* 1991; Yoon *et al.* 1994; Delputte and Nauwynck 2004). PRRSV infects pigs through the mucosal surface of the respiratory tract (Ostrowski *et al.* 2002). After infection, large amounts of nonneutralizing antibodies (NNA) soon appear, while a low titre of neutralizing antibodies (NAs) is detectable not sooner than 3 weeks post-infection. NAs are believed to play a critical role in protection against PRRSV infection (Yoon *et al.* 1994; Pirzadeh and Dea 1998). Previous studies have shown that monoclonal antibodies (MAbs) recognizing the viral GP5 protein neutralize PRRSV more effectively than those recognizing the other viral proteins (Weiland *et al.* 1999), suggesting that GP5 is the primary target for NAs to deactivate the virus. Furthermore, Ostrowski *et al.* (2002) identified a GP5 neutralizing epitope that is associated with PRRSV neutralization. Thus, the induction of NAs against GP5 neutralizing epitopes (GP5-NE) is critical for the control of PRRSV.

Over the last decade, the application of transgenic plants producing edible therapeutic proteins or vaccine antigens against human and animal diseases has become a promising approach (Carrillo *et al.* 1998; Castanon *et al.* 1999; Yuki *et al.* 2001; Yusibov *et al.* 2002; Kim and Langridge 2003; Ma *et al.* 2004; Wigdorovitz *et al.* 2004; Dus Santos *et al.* 2005; Pogrebnyak *et al.* 2005; Li *et al.* 2006; Golovkin *et al.* 2007; Jiang *et al.* 2007; Nochi *et al.* 2007; Sharma *et al.* 2008; Wang *et al.* 2008a; Skarma and Sharma 2009). It has been shown that after oral administration, the plant-produced antigen proteins have access to the mucosal cells and induce antigen-specific immune response in both systemic and mucosal compartments. This provides a safe method for inducing protective immune responses without injection-related hazards (Pogrebnyak *et al.* 2005). Thus, the plant system offers practical, biochemical, economic and safety advantages compared with conventional systems for the production of antigens (Kermode 2006; Streatfield 2006; Wang *et al.* 2008). PPRSV establishes its infection via mucosal routes. Production of oral vaccines to induce specific mucosal antibody response may represent the most effective approach to preventing PPRSV infection (**Fig. 1**). In this mini review, we summarize our progress in developing a pant-based vaccine against PRRSV and highlights future prospects for this research.

EXPRESSION OF CHOLERA TOXIN B, AN ADJUVANT PROTEIN IN TRANSGENIC PLANTS

Induction of mucosal immunity by oral route of immunization with plant-derived antigens is a cost-effective and promising approach for preventing mucosal infections to treat various infectious-immunopathological disorders (Holmgren *et al.* 2003). There are several advantages to using a mucosal route of vaccination over a parenteral route. The most important one is that the vast majority of infections take place or initiate at the mucosal surface. PPRSV is an example of infectious pathogens that cause such infections. Against such infectious pathogens, the most effective protection is to induce a topical immunity. To develop a plant-based, mucosal vaccine against such pathogens, the use of an adjuvant is essential to ensure that the vaccine is effectively delivered into the mucosal site for the induction of appropriate mucosal immune response.

Mucosal adjuvants

The two most widely used mucosal adjuvants are the heat labile toxin (LT) of enterotoxigenic *Escherichia coli* and the cholera toxin (CT) of *Vibrio cholerae* (Holmgren *et al.* 2003; Rigano *et al.* 2003; Streatfield 2006). Both LT and CT are powerful adjuvants, consisting of a non-toxic homopentamer of B subunits and a single toxic A subunit. The A subunit has two protein domains, one responsible for the enzymatic activity that ADP-ribosylates the Gc protein of adenylate cyclase and the other for the association of the A subunit with the B subunit (de Haan and Hirst 2000). The B subunit forms a pentamer with five identical monomers that binds to GM1 gangliosides on the surface of the mucosal epithelial cells and thus target the holotoxin to the mucosal lymphoid tissues. Since the A subunit is toxic, the B subunits of CT and LT (CTB and LTB) have been extensively studied for their function as carrier molecules for foreign proteins and their ability to facilitate immune response to the co-administered antigens (Holmgren *et al.* 2003). It has been shown that although LTB and CTB are close homologues, sharing as high as 80% identity at nucleotide or amino acid levels, they have distinct biochemical and immunological differences (Rigano *et al.* 2003). LTB can bind to a wider range of receptors that contain galactose (Gal) than CTB, which only binds to sugar-lipid GM1 gangliosides (Zhang *et al.* 1995; Bowman and Clements 2001). Both of them have exhibited a similar adjuvant activity (Dertzaugh and Elson 1993; Pascual 2007). However, it seems that CTB has been preferred in plant expression systems as shown in a number of publications (Sun *et al.* 1994; Arakawa *et al.* 1998; Daniell *et al.* 2001; Wang *et al.* 2001; Jani *et al.* 2002; Kim and Langridge 2003; Jani *et al.* 2004; Li *et al.* 2006; Jiang *et al.* 2007; Nochi *et al.* 2007; Sharma *et al.* 2008).

Expression of CTB in transgenic plants

To test if CTB is expressible in our expression system, we cloned the CTB gene into a plant transformation vector, i.e., pCaMterX, under the control of the double 35S promoter (Wang *et al.* 2008a). The plant expression vector containing the gene cassette of 35S-35S-promoter::CTB::Nos-terminator was transformed into non-nicotine and low-alkaloid *Nicotiana tabacum cv.* 81v9 (Wang *et al.* 2008a). Eighteen transgenic tobacco lines were generated. The presence of the CTB gene in the putative transgenic lines was confirmed by PCR. Abundant CTB mRNAs were detected in total RNA isolated from these transgenic lines by Northern blot. Western blot analysis showed that CTB formed a pentamer in transgenic plants. Quantitative enzyme-linked immunosorbent assay (ELISA) showed that the expression level of the CTB protein varied from 0.001 to 0.15% of the total soluble protein (TSP) in leaf tissues of transgenic plants with an average of about 0.08% (**Fig. 2**). GM1-ELISA binding assay indicated that CTB in the protein extracts from transgenic plants efficiently bound to the mucosal GM1 receptor, suggesting the plant-produced CTB protein is biologically active. These results are consistent with several recent reports (Li *et al.* 2006; Jiang *et al.* 2007; Sharma *et al.* 2008).

To optimize our expression system, we tested different promoters (Nos and tCUP), deleted unnecessary sequence in the transformation vector, added *Alfalfa mosaic virus* (AMV) leader sequence before the start codon, and in-

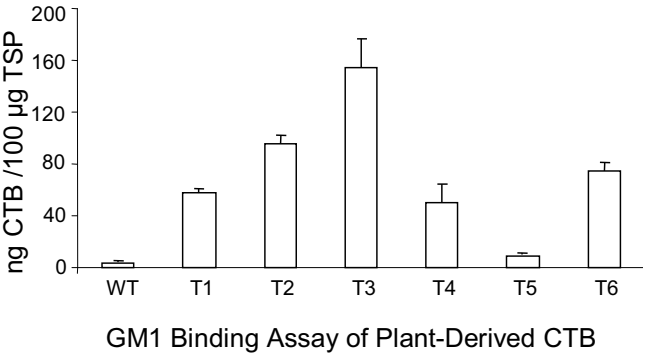

Fig. 2 GM1 binding assay of the plant-derived CTB protein.

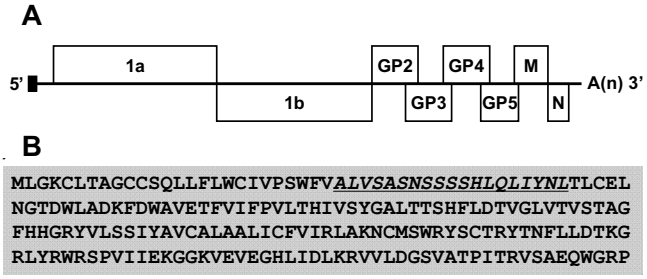

Fig. 3 Production of GP5 as an antigen subunit in plants. (**A**) Schematic representation of arterivirus genomic organization. It is modified based on Albina (1997). (**B**) The amino acid sequence of the GP5 protein. The sequence is retrieved from GenBank with an accession number AAC41227 (Gagnon and Dea 1998). A neutralizing epitope identified by Ostrowski *et al*. (2002) and used in this study is underlined and italicized.

cluded KDEL (ER retention signal) before the stop codon. We found that the double 35S promoter with the AMV leader sequence and ER retention signal helped increase the CTB yield by approximately 30%. This optimized system was suitable for the expression of a vaccine antigen against PRRSV as was successfully adapted for the production of recombinant human interleukin-13 in transgenic plants (Wang *et al.* 2008a, 2008b).

PRODUCTION OF PRRSV ANTIGENS IN TRANSGENIC PLANTS

Selection of a PRRSV antigen for expression

With the availability of an optimized expression system and an adjuvant, the next step was to select a proper antigen to express in plants. As briefly described in introduction, PRRSV produces 9 viral proteins (**Fig. 3**). Three of them are major structural proteins including the envelope glycoprotein GP5 (25 kDa), the non-glycosylated membrane protein M (18 kDa) and the nucleocapsid protein N (14 kDa) (Mardassi *et al.* 1996; Delputte and Nauynck 2004; Kheyar *et al.* 2005). These structural proteins are closely associated. GP5 and M proteins form heterodimers (Mardassi *et al.* 1996). After infection, these structural proteins can induce the production of large amounts of nonneutralizing antibodies (NNA) and a low titer of neutralizing antibodies (NAs). It is NAs that play a critical role in protection against PRRSV infection (Yoon *et al.* 1994; Pirzadeh and Dea 1998). Previous studies have shown that circulating antibodies in PRRSV-infected pigs responsible for viral neutralization in cell cultures are mainly directed against GP5 (Gonin *et al.* 1999). Genetic immunization of pigs with a DNA vector expressing GP5 induces the production of low titers of neutralizing antibodies to PRRSV (Pirzadeh and Dea 1998). Extensive analyses using mice as an animal model suggest that PRRSV GP5 in principle is a major target for NAs to deactivate the virus (Weiland *et al.* 1999). Furthermore, Ostrowski *et al.* (2002) identified the neutralizing epitope (BA) and nonneutralizing epitopes (NNE) in the GP5 protein (**Fig. 3**). All these findings indicate that induction of NAs against GP5 is crucial for the control of PRRSV. GP5 logically becomes the first choice of the oral vaccine antigens.

Expression of the full-length GP5 protein

The full-length GP5 (a kind gift from C. Gagnon, INRS-Institut Armand-Frappier, Laval, Canada) or its fusion with CTB was cloned and inserted into our optimized vector. A number of transgenic tobacco plants were generated. Though high levels of GP5 mRNA were evident in transgenic plants by Northern blot, the GP5 or CTB-GP5 recombinant protein was hardly detectable by Western blot, suggesting a very low level of the GP5 recombinant protein in transgenic plants. These results are consistent with findings resulting from an earlier independent study in transgenic tobacco and alfalfa by J. Brandle *et al.* (pers. comm.). The low GP5 accumulation was not improved by transformation of a codon-optimized synthesis GP5 gene (J. Brandle, pers. comm.). The full-length GP5 seems recalcitrant to accumulation in transgenic plants. It is not clear if this is due to a possible abortion of translation, a very short turn-over time, or the toxicity of GP5 in plant cells.

Expression of the partial G5 protein

As discussed above, GP5 consists of NA and NNA epitopes. It is the NA epitope that induces the production of NAs which effectively deactivate PRRSV. Partial GP5 cDNA fragments from three representative Canadian PRRSV isolates, each of which contains 147 nucleotides encoding a neutralizing epitope (GP5-NE), were synthesized and cloned into the optimized expression vector to constitute the following cassette: 35S-35S::AMV-leader-sequence::CTB-(GP5-NEa)-(GP5-NEb)-(GP5-NEc)-(KDEL)::Nos-terminator. This vector was transformed into tobacco plants. The quantification of the recombinant protein with ELISA indicated that the expression level of CTB-GP5-NE was in the range of 0.003 to 0.087% of TSP from transgenic tobacco. Further, GM1-ELISA binding assay confirmed that the plant-produced CTB-GP5-NEabc was biologically active. A transgenic tobacco line expressing the highest level of CTB-GP5-NEabc is ready for animal feeding test.

VIRUS-DIRECTED EXPRESSION OF GP5 IN PLANTS

Plant viruses have the ability to produce and accumulate high levels of viral proteins in infected plants. Thus, plant viruses have great potential to be developed into an expression vector for the production of heterologous proteins in plants (Gleba *et al.* 2004). Indeed, during the past few years, several viral vectors have been reported (Masuta *et al.* 2000; Mor *et al.* 2003; Beauchemin *et al.* 2005; Lindbo 2007; Sainbury *et al.* 2008). In some cases, expression levels of the recombinant protein can reach as high as 10% of TSP (Dohi *et al.* 2006). These viral vectors were not adapted for our research due to either limited host ranges or safety concerns.

To boost the GP5 expression level and domesticate legume plants as host plants that are major pork feed for proteins, we initiated the development of a *Soybean mosaic virus* (SMV)-based vector for the production of GP5. Two Canadian SMV isolates were collected. Their viral genome (about 9.5 kb) was completely cloned and sequenced (Gagarinova *et al.* 2008). A full-length virus strategy was used to construct the SMV expression vector. A yellow fluorescent protein (YFP) was inserted into the junction of P1 and HC-Pro. Strong YFP signals were found in the plants infected by this clone. GP5, GP5-NEabc, CTB-GP5 and CTB-GP5-NEabc were inserted in the SMV viral vector. Currently, expression levels of GP5 in soybeans infected

with these SMV-GP5 derivatives are being evaluated.

PRODUCTION OF GP5 USING OTHER PLANT SYSTEMS

In addition to the constitutive, nuclear expression and viral expression vector systems described above, other plant systems may also be adapted for the expression of GP5 in plant hosts. These include tissue-specific expression such as cereal seeds (Hood *et al.* 2003; Nochi *et al.* 2007), inducible expression such as chemical inducible systems (Padidam 2003), plant cell suspension cultures (Fischer *et al.* 1999), and chloroplasts (Grevich and Daniell 2005). Among them, the chloroplast system has attracted more and more attention (Daniell *et al.* 2005). Transgenic chloroplasts allow the high-yield production of vaccine antigens of interest. It has been reported that in transgenic tobacco chloroplasts, biologically active CTB account for as high as 4% of TSP (Daniell *et al.* 2001). In the case of a rotavirus VP6 protein, the expression level in transgenic tobacco chloroplasts varies from 0% to 3% of TSP, dependent on the promoter used (Birch-Machin *et al.* 2004). It seems that the vaccine accumulation in chloroplasts largely depend on its regulatory sequences and the toxicility of the vaccine (Daniell *et al.* 2005). It would be interesting to determine if transgenic chloroplasts allow the accumulation of high levels of GP5.

CONCLUSION REMARKS AND FUTURE DIRECTIONS

Over the last decade, a number of proof-of-principle studies have been conducted to test plants as new bioreactors for vaccine production. Some of the subunit vaccines investigated to date have been exceptionally successful and moved forward for clinical trials and scale-up production on the commercial basis. However, overall there are still several major limitations preventing from the commercialization of the plant-produced vaccines. A major technical bottleneck is the low yield of certain antigen proteins, especially those of viral origin (Wu *et al.* 2004; Golovkin *et al.* 2007). To overcome this impediment, diverse expression systems such as plant virus-directed expression and chloroplast expression systems have been developed, albeit it is unknown why some of antigen proteins accumulate at a high level in one expression system but very little in another system. Other long-standing challenges include issues of environmental impact, biosafety and risk assessment.

We attempted to use transgenic plants to produce GP5 as a subunit vaccine against PRRSV. Although the CTB-GP5-NE was expressible in tobacco plants and the plant-produced CTB-GP5-NE was biologically active, the expression level was low. We developed an SMV-based viral vector to enhance the expression of GP5 in legume plants. Unlike non-food plants such as tobacco, legume plants expressing GP5 may directly be fed as edible subunit vaccines to pork. Currently we are focused on the enhancement of the GP5 production and looking forward to the initiation of the animal feeding test. Our study has set a first step toward the utilization of plants for the production of subunit vaccines against PRRSV.

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