Plant synthetic GP4 and GP5 proteins from porcine reproductive and respiratory syndrome virus elicit immune responses in pigs

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Abstract

Main conclusion **We demonstrated successful overexpression of porcine reproductive and respiratory syndrome virus (PRRSV)-derived GP4D and GP5D antigenic proteins in** *Arabidopsis***. Pigs immunized with transgenic plants expressing GP4D and GP5D proteins generated both humoral and cellular immune responses to PRRSV.**

Porcine reproductive and respiratory syndrome virus (PRRSV) causes PRRS, the most economically significant disease affecting the swine industry worldwide. However, current commercial PRRSV vaccines (killed virus or modified live vaccines) show poor efficacy and safety due to concerns such as reversion of virus to wild type and lack of cross protection. To overcome these problems, plants are considered a promising alternative to conventional platforms and as a vehicle for large-scale production of recombinant proteins. Here, we demonstrate successful production of recombinant protein vaccine by expressing codon-optimized and transmembrane-deleted recombinant glycoproteins (GP4D and GP5D) from PRRSV in planta. We generated transgenic *Arabidopsis* plants expressing GP4D and GP5D proteins as candidate antigens. To examine immunogenicity, pigs were fed transgenic *Arabidopsis* leaves expressing the GP4D and GP5D antigens (three times at 2-week intervals) and then challenged with PRRSV at 6-week post-initial treatment. Immunized pigs showed significantly lower lung lesion scores and reduced viremia and viral loads in the lung than pigs fed *Arabidopsis* leaves expressing mYFP (control). Immunized pigs also had higher titers of PRRSV-specific antibodies and significantly higher levels of pro-inflammatory cytokines (TNF-α and IL-12). Furthermore, the numbers of IFN-γ[+]-producing cells were higher, and those of regulatory T cells were lower, in GP4D and GP5D immunized pigs than in control pigs. Thus, plant-derived GP4D and GP5D proteins provide an alternative platform for producing an effective subunit vaccine against PRRSV.

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Introduction

Porcine reproductive and respiratory syndrome (PRRS) is an economically important disease that causes major losses to swine industry worldwide, accounting for $664 million in USA alone (Holtkamp et al. 2013; Neumann et al. 2005). The disease is characterized by acute reproductive failure in sows and respiratory disease in pigs of all ages (Hanada et al. 2005). The PRRS virus (PRRSV), the causative agent of the disease, is a positive-sense RNA virus belonging to the family *Arteriviridae* (order, *Nidovirales*) (Cavanagh 1997). PRRSV comprises a 15.4-kb genome encoding ten open reading frames (ORFs), of which ORF1a and 1b encode the non-structural proteins (nsps), while the

remaining eight (ORFs 2a, 2b, 3, 4, 5a, 5, 6, and 7) encode structural proteins (Johnson et al. 2011; Snijder et al. 2013). The virus was first isolated in Western Europe in 1991 and then subsequently in North America and other swine-rearing countries (Lager et al. 2014). Based on geographic origin and genetic composition, PRRSV is classified into two major genotypes: the European type (type I) and the North American type (type II) (Nelsen et al. 1999). The two genotypes share less than 70% nucleic acid homology across the entire genome, resulting in antigenic variability and immunological diversity (Gimeno et al. 2011; Kim et al. 2007; Murtaugh et al. 1995). Currently, modified live virus (MLV) vaccines are used to control PRRSV. However, due to antigenic and genetic diversity among PRRSV isolates, MLV vaccines do not provide effective cross protection. Furthermore, there are increased concerns regarding the safety of these vaccines due to rapid virus reversion to virulence during replication in pigs (Charerntantanakul 2012; Hu and Zhang 2014; Opriessnig et al. 2002). PRRS-killed virus vaccines and recombinant vaccines have also been developed but do not confer improved protection against homologous strains (Kim et al. 2011; Zuckermann et al. 2007). Thus, due to the shortcomings of the current vaccines, efforts are being made to develop vaccines that are more effective against PRRSV. Plants offer cost-effective platforms for vaccine production. Plant-made subunit vaccines are heat stable, are not contaminated with animal pathogens, and can be engineered to contain multiple antigens (Davoodi-Semiromi et al. 2010; Hefferon 2013). Plant-based antigens can be fed directly to animals or humans without the need for purification or processing. Plant-made subunit vaccines for the production and delivery of antigen proteins are economical, safe, and effective alternatives (Mason et al. 2002).

Previously, plant-based anti-PRRSV vaccines expressing different antigenic proteins derived from the virus were developed. Glycoprotein 5 (GP5) was used successfully as an antigen and expressed in banana (Chan et al. 2013), potato (Chen and Liu 2011), and tobacco (Chia et al. 2010) plants. Moreover, M and N protein antigen candidates were also expressed in corn calli (Hu et al. 2012) and soybean (Vimolmangkang et al. 2012), respectively. Recently, *Arabidopsis* plant seeds expressing GP3, GP4, and GP5 proteins from PRRSV were reported (Piron et al. 2014). Furthermore, *Arabidopsis* plants are edible by mice and pigs without adverse effects; therefore, the plants can be used as a model system for developing subunit vaccines.

Here, we aimed to develop a plant-based subunit vaccine against PRRSV by expressing codon-optimized and transmembrane (TM)-deleted antigenic proteins (GP4D and GP5D) in *Arabidopsis* plants. For this, we used ER-targeting vector systems to generate transgenic *Arabidopsis* plants expressing high levels of GP4D and GP5D proteins. We then showed that pigs fed GP4D and GP5D transgenic

Arabidopsis leaves, followed by challenge with PRRSV, exhibited a PRRSV-specific immune response. Thus, GP4D and GP5D proteins expressed in transgenic *Arabidopsis* provide effective protective immunity against PRRSV.

Materials and methods

Plant materials and plant transformation

Arabidopsis thaliana (ecotype Columbia) was used to express recombinant GP4 and GP5 (GP4D and GP5D) proteins from PRRSV. Four to five weeks after sowing the seeds in soil, plants were transformed with *Agrobacterium tumefaciens* strain GV3101 using the floral dip method (Clough and Bent 1998). Transformants were selected on soil by treating with Basta (glufosinate-ammonium) three times at 3 day intervals. For pig immunization, transgenic *Arabidopsis* T2 (about 100 plants) from line 3–2 of GP4D and line 1–1 of GP5D were used. Plants were grown under long-day growth conditions (14-h light/10-h dark cycle) at 22 °C.

Gene and vector constructs

DNA sequences encoding the GP4 and GP5 subunit proteins of PRRSV were derived from the North America type JA142 strain (GenBank accession number: AY424271.1). The truncated recombinant *GP4D* and *GP5D* genes [lacking the TM domain and transit signal peptide sequences (SPs)] were synthesized after codon optimization (Supplementary Fig. S1). The optimized nucleotide sequences of *GP4D* and *GP5D* comprised 483 and 348 bp, encoding 161 and 116 amino acid sequences, respectively. The sequences encoding GP4D and GP5D were cloned into the binary vector pGreenII0229, which comprises the 5′-leader sequence (called Ω) region of tobacco mosaic virus (TMV), a double enhancer CaMV *35S* (d35S) promoter, a nopaline synthase (NOS) terminator, and a selectable marker gene for kanamycin resistance (*nptII*) (Fig. 1b). To obtain high-level expression of the transgenes in plants, the recombinant GP4D and GP5D proteins were targeted to the endoplasmic reticulum (ER) by incorporating an N-terminal transit peptide (VTS) and the C-terminal ER retention signal KDEL (K). The VTS is a vacuolar targeting signal derived from *sporamin A* (GenBank accession number: AFR60855.1) in sweet potato. To facilitate detection of GP4D and GP5D proteins, a Flag-tag was attached to the C-terminus.

Western blot analysis

Western blot analysis of leaf samples collected from transgenic *Arabidopsis* plants was performed as described by Jeong et al. (2016). Briefly, protein samples (20–30 µg)
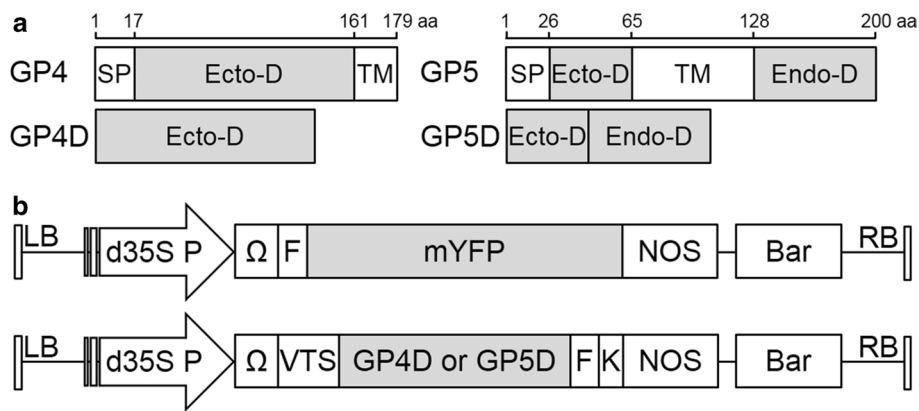
Fig. 1 Schematic representation of the original and synthesized formats of the GP4 and GP5 genes and the expression cassette. **a** GP4 and GP5 are the full-length forms of the genes. GP4D and GP5D are the synthesized forms lacking the TM and SP following codon optimization. *aa* position of the amino acids coding the gene, *SP* transit signal peptide sequence, *Ecto-D* ectodomain, *Endo-D* endodomain, *TM* transmembrane domain. **b** Plant expression vector, pGreenII0229; *d35S P* dual *35S* promoter, *Ω* TMV leader sequence, *F* FLAG-tag, *NOS* 3′-NOS terminator, *VTS* vacuolar targeting signal, *K* (*KDEL*) ER retention signal, *Bar* Basta resistance gene, *LB* left border, *RB* right border

were separated on 12% SDS-PAGE gels and electrotransferred to polyvinylidene fluoride (PVDF) membranes using a semi-dry blotting apparatus (Bio-Rad). The membrane was then blocked for 2 h in TBS-T buffer containing 3% (W/V) non-fat dried milk (Santa Cruz), probed with a monoclonal anti-Flag M2 antibody (1:10,000 dilution; F3165, Sigma-Aldrich), and visualized using the SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific).

Glycan analysis

Glycan analysis was performed according to the manufacturer's protocol (NEB, N-glycosidase F and endoglycosidase H, cat. nos. P0704L and P0702L, respectively). Total soluble protein (TSP) from plant leaves was treated with peptide N-glycosidase F (PNGase F) and recombinant endoglycosidase H (endo H) to analyze glycosylation of GP4 and GP5 proteins. Furthermore, 1 µl of denaturing buffer and distilled water was added to 5 µl of TSP (total reaction volume, 10 µl). Next, the sample was incubated at 100 °C for 10 min to allow protein denaturation. For PNGase F digestion, 2 µl of G7 buffer, 2 µl of NP40, and 2 µl of PNGase F were added to the sample. For endo H digestion, 2 µl of G5 buffer and 2 µl of endo H were added. Finally, ddH$_2$O was added up to 20 µl, after which samples were incubated for 1 h at 37 °C prior to western blot analysis.

Cells and viruses

MARC-145 cells were used for virus propagation and for the virus assays. MARC-145 cells were maintained at 37 °C/5% CO$_2$ in RPMI-1640 medium (Gibco® RPMI 1640, Invitrogen, CA, USA) supplemented with heat inactivated 10% fetal bovine serum (FBS, Invitrogen, CA USA), 2 mM L-glutamine, and Antibiotic–Antimycotic 100× [Anti–anti, Invitrogen; 1× solution contains 100-IU/ml penicillin, 100-µg/ml streptomycin, and 0.25-µg/ml Fungizone® (amphotericin B)]. The PRRSV strain JA142 was used to challenge the animals.

Immunization and assessment of immunogenicity

Ten 4-week-old-specific pathogen-free piglets were purchased from a PRRSV-negative farm and randomly assigned to two groups ($n = 5$). Pigs in the control group were fed (via feeding tubes) with a fine powder of control *Arabidopsis* (referred to as mYFP transgenic plants) leaves (20-g fresh weight, FW) at three time points (days 0, 14, and 28). The second group received a powdered mix of transgenic *Arabidopsis* (referred to as GP4D/GP5D transgenic plants) leaves (20-g FW) containing about 1 mg of GP4D and 1 mg of GP5D protein. After immunization, pigs received regular water and commercial feed. All animals in each group were then intra-nasally challenged with JA142 strain (1×10^3 TCID$_{50}$/ml) 2 weeks after the third feed [i.e., at 42-day posttreatment (dpt)]. All pigs were bled at 0, 7, 14, 21, 25, 30, 42, 49, and 56 dpt to collect serum for cytokine analysis and whole blood for porcine peripheral blood mononuclear cell (PBMC) isolation. These cells were used to assess cytokine mRNA transcript levels and for flow cytometry. Sera were separated immediately after bleeding and stored at − 80 °C. All pigs were euthanized at 56 dpt and subjected to pathological evaluation. To evaluate gross and microscopic lung lesions, each lung lobe was scored according to the percentage of lung consolidation (Halbur et al. 1995) and interstitial pneumonia, respectively, caused by PRRSV infection.

Scoring of microscopic lung lesions was recorded on a scale of 0–3 as follows: 0, no lesion; 1, mild interstitial pneumonia; 2, moderate multifocal interstitial pneumonia; 3, severe interstitial pneumonia. Lung tissues were collected from each pig and stored at − 80 °C until examined. All animal experiments were approved by the Chonbuk National University Institutional Animal Care and Use Committee (Approval Number: 2012-0025).

Reverse transcriptase-PCR analysis

Viremia was examined on days 42, 49, and 56 dpt, and the residual viral load in the lungs after euthanasia (at 56 dpt) was quantified. The MagMAX Viral RNA extraction kit (Ambion, CA, USA) and total RNA extraction kit (GeneAll, Seoul, Korea) were used to extract viral RNA from serum and lung samples, respectively. Virus levels in serum and lungs were measured using real-time reverse transcription-polymerase chain reaction (RT-PCR) employing Taqman® chemistry (Applied Biosystems, CA, USA). Primer Express® software V 3.0 (Applied Biosystems, USA) was used to design the primers and an MGB fluorescent probe specific for a conserved region of ORF7. The sequences of primers and probe are as follows: Forward Primer, TGTCAGATT CAGGGAGRATAAGTTAC; Reverse Primer, ATCARG CGCACAGTRTGATGC; and Probe, 6FAM TGTGGAGTT YAGTYTGCC. Real-time RT-PCR was performed using a one-step reverse transcriptase kit (AgPath-IDTM One-Step RT-PCR Kit, Ambion, USA) and the 7500 Fast Real-Time PCR system (Applied Biosystems, TX, USA). The following cycling conditions were used: (a) reverse transcription for 10 min at 45 °C; (b) a 10-min activation step at 95 °C; and (c) 40 cycles of 15 s at 95 °C and 45 s at 60 °C. Samples with a threshold cycle (Ct) of 35 cycles or less were considered positive. A standard curve constructed from known virus titers was used to calculate the amount of PRRSV in each sample by converting the Ct value to virus titer (50% tissue culture infectious dose, $TCID_{50}$/ml).

Assessment of PRRSV-specific antibody responses

The titers of an indirect fluorescent antibody (IFA) were determined at 42, 49, and 56 dpt to observe changes in antibody titers after treatment and challenge. The 96-well plates containing MARC-145 cell monolayers were inoculated with 100 µl of JA142 strain (10^4 $TCID_{50}$/ml) to prepare the antigen. Infected cells were fixed with cold 80% acetone after 20 h incubation at 37 °C. Four wells per plate were inoculated with virus-free cell culture medium to act as a control. All plates were dried and stored at − 20 °C until use. The PRRS viral antigen was confirmed by immunofluorescence microscopy using a PRRSV-specific monoclonal antibody, anti-PRRS NC Mab 4A5 (Median Diagnostic,

Gangwondo, Korea), labeled with FITC-conjugated goat anti-mouse IgG (Bethyl Laboratories, TX, USA). For each serum sample, a series of twofold dilutions were prepared in 0.01-M phosphate-buffered saline (1× PBS, pH 7.2), and 50 µl of each diluted sample was added to wells containing viral antigen. The plates were then incubated at 37 °C for 1 h. After washing the samples with 0.01-M PBS (pH 7.2), the antigen antibody reaction was visualized using goat anti-pig IgG conjugated to FITC (Bethyl Laboratories, USA). The plates were then incubated at 37 °C for 1 h and later observed under a fluorescence microscope to examine the PRRSV-specific antibody titer in each serum sample. The titer was calculated as the reciprocal of the highest dilution in which specific fluorescence was detected.

Isolation of PBMCs

PBMCs were isolated from 5 ml of blood (collected in lithium-heparin-containing vacutainers) using the density gradient method on Histopaque-1077® solution (Sigma, MO, USA), according to the manufacturer's instructions. Whole blood was collected from all pigs on days 7, 14, 30, 42, and 56. After brief stratification on Histopaque-1077® solution (blood: Histopaque ratio, 1:1), cells were centrifuged at 400×*g* for 30 min. The purified PBMCs were collected and washed twice with sterile PBS (pH 7.0) supplemented with 1% FBS (Gibco, USA), and then re-suspended in 0.5 ml of sterile PBS. The viability and number of cells were evaluated by diluting them 100× in 1× PBS. They were then mixed with 0.4% Trypan Blue (1:1 ratio, v/v) and counted using a Countess™ Automated Cell Counter (Invitrogen, USA). Cells were diluted to 1×10^6 cells/ml in RPMI, and 1 ml per well was seeded into 24-well plates (BD Falcon, USA) and incubated at 37 °C in a humidified 5% CO_2 incubator for 72 h.

Flow cytometry

PBMC isolation was followed by determination of the percentage and phenotype of the immune cell populations using multicolor immunostaining of single-cell suspensions (performed on the same day as isolation). The T cell response of each pig was evaluated by assessing expression of markers of regulatory T cells (CD4+CD25+FoxP3+) (Tregs), Th1 responses (CD4+IFN-γ+), and cytotoxic T cells (CD8+IFN-γ+). Cells were stained with a monoclonal antibody directly conjugated to a specific fluorochrome or biotin or with a purified antibody specific for porcine immune cell surface markers.

Isolated PBMCs were further treated for 2 min with 1× red blood cell lysis buffer (eBiosciences, CA, USA) and washed twice with 1× PBS, and viability and cell number were evaluated. Briefly, 1×10^6 purified PBMCs per sample

were re-suspended in fluorescence-activated cell sorting (FACS) buffer (2% FBS in phosphate-buffered saline and 0.02% sodium azide) and two aliquots of each sample were plated into two separate U-bottom 96-well plates (1×10^6 cells/200 µl/well). Three aliquots of cells in different plates were pelleted by centrifugation (at $400g$, 4 °C, for 5 min), and the supernatant was carefully discarded. Subsequently, the cells in one plate were stained with either CD4α-PE (Clone 74-12-4; BD Biosciences, USA) or anti-porcine CD25 (clone K231.3B3; Serotec, Raleigh, NC, USA) in cold FACS buffer and incubated in the dark at 4 °C for 30 min. Next, cells were washed three times with cold FACS buffer (200 µl/well) and stained with an allophycocyanin-conjugated rat anti-mouse IgG1 Ab (Clone RMG1-1; Bio-Legend, San Diego, USA) as a secondary antibody against anti-CD25, followed by washing as described above. Afterwards, cells were fixed with cold fixation/permeabilization buffer (eBiosciences) at 4 °C for 30 min, followed by staining with FoxP3-FITC (Clone FJK-16s; eBiosciences, San Diego, USA) in cold permeabilization buffer (eBiosciences) at 4 °C for 30 min. The cells were then washed twice with cold permeabilization buffer (200 µl/well) and re-suspended in 100 µl of FACS buffer. Simultaneously, the two other sets of cells were treated with 1× cell stimulation cocktail (eBiosciences) plus 1× brefeldin A (eBiosciences) in cRPMI medium and incubated at 37 °C in a 5% CO_2 humidified chamber for 5 h. Afterwards, one cell set was stained with CD8α-FITC (Clone 76-2-11; BD Biosciences) and the other with CD4α-PE in cold FACS buffer in the dark at 4 °C for 30 min. The cells were then washed twice with cold FACS buffer and fixed with IC fixation buffer (eBiosciences) at 4 °C for 30 min. After washing twice with permeabilization buffer (200 µl/well), the cells were stained with IFN-γ-PerCP-CyTM 5.5 (Clone P2G10; BD Biosciences) in cold permeabilization buffer at 4 °C for 30 min. Next, the cells were washed twice with permeabilization buffer and finally re-suspended in 100 µl of FACS buffer. Next, 100,000 events (gated by forward and side scatter) per sample were acquired using an Accuri C6 flow cytometer (BD Biosciences, USA) and data were analyzed using BD CFlow®Plus software v. 1.0.227.4 (BD Biosciences, USA). The percentage of activated, regulatory, TH1, and cytotoxic T cells was then calculated.

Cytokine ELISA

A commercial sandwich ELISA (DuoSet® ELISA, R&D Systems, MN, USA) was used to measure TNF-α and IL-12 protein levels in serum. Briefly, the wells of a 96-well plate were coated with anti-pig-IL-12 (4 µg/ml) or anti-pig TNF-α (8 µg/ml) antibodies diluted in PBS. The plates were then incubated overnight at room temperature (RT) and washed three times with wash buffer. The wells of the 96-well plate were blocked with 300 µl of reagent diluent (R&D Systems, #DY995, MN, USA) and incubated at RT for 1 h. After washing three times, 100 µl of sample or standard, diluted with reagent diluent for IL-12, or reagent diluent [1% BSA in PBS, pH 7.2–7.4, 0.05% Tween® 20 (Promega, WI, USA)] in Tris-buffered saline [20 mM Trizma base, 150 mM NaCl, pH 7.2–7.4, 0.2 µm filtered] for TNF-α, was added to the plates. The washing step was repeated, and secondary antibody (100 µl per well, diluted in appropriate reagent diluent) was added for 2 h. After washing, 100 µl of a working dilution (1/200) of streptavidin–horseradish peroxidase (HRP) was added to each well for 20 min. The washing step was then repeated, and 100 µl of 3,3′,5,5′-tetramethylbenzidine (TMB) solution (R&D Systems) was added. Plates were incubated in the dark for 20 min at RT to allow the development of color. The reaction was stopped by adding 50 µl of 2-N H_2SO_4 per well, and optical density (O.D.) was measured at 450 nm within 30 min. The results were analyzed using SoftMax Pro 5.3 microplate data software (Molecular Devices, CA, USA).

Data analysis

GraphPad Prism 5.0.2 (GraphPad, San Diego, CA, USA) was used to construct graphs. Statistical analysis was performed using SPSS Advanced Statistics 17.0 software (SPSS, Inc., Chicago, USA). The Mann–Whitney U test was used to analyze the significance of differences between groups.

Results

Expression of recombinant GP4D and GP5D proteins in *Arabidopsis* plants

The PRRSV glycoprotein GP5, a major envelope glycoprotein, is one of the key immunogenic proteins of PRRSV used for the vaccine development (Kheyar et al. 2005; Pirzadeh and Dea 1998). GP4, a minor envelope glycoprotein interacting with GP5, is also a critical viral envelope protein containing a highly variable neutralizing epitope (Costers et al. 2010; Das et al. 2010). Thus, we chose GP4 and GP5 as candidate antigens to develop plant-based PRRSV vaccines. Both full-length sequences of GP4 and GP5 contain a transit SP and a TM domain (Fig. 1a). We synthesized codon-optimized *GP4* and *GP5* genes lacking the TM and SP sequences (referred to as GP4D and GP5D, respectively; Fig. S1) and cloned them into the plant expression vector pGreenII0229, which includes the *d35S* promoter, Ω, VTS, and the ER retention signal for high-level expression of GP4D and GP5D proteins (Fig. 1b). We then generated transgenic *Arabidopsis* plants overexpressing the synthetic

GP4D and *GP5D* genes. Transgenic *Arabidopsis* T1 plants were selected using Basta and at least 20 Basta-resistant plants were obtained from the T1 generation. Among these, GP4D and GP5D positive transgenic lines (11 each) were confirmed by Western blot analysis (Fig. S2). T2 seeds were harvested from T1 plants; line 3–2 of GP4D and line 1–1 of GP5D exhibited relatively high protein expression compared to the other lines. Transgenic *Arabidopsis* T2 plants were further selected via Basta treatment, and GP4D and GP5D transgenic lines (12 each) were confirmed by Western blot analysis (Fig. 2). In all leaf samples from line 3–2 of GP4D, signal bands were detected at around 30 kDa and putative dimer patterns were also observed (Fig. 2a). For GP5D, signal bands were observed in all 12 lines around 23 kDa and dimer patterns were also detected (Fig. 2b). These results show that the recombinant GP4D and GP5D proteins were successfully expressed in transgenic *Arabidopsis* plants. When we measured the expression levels of GP4D and GP5D proteins and compared them with that of a multiple tag marker used as a reference, we found that they comprised about 1.66% of TSP in transgenic *Arabidopsis* plants.

Glycosylation status of antigens produced in *Arabidopsis* leaves

The recombinant GP4D and GP5D proteins had molecular weights of 30 and 23 kDa, respectively, on SDS-PAGE, but their predicted sizes are 18 and 15 kDa, respectively. The difference is probably due to plant-derived glycosylations. Proteins retained in the ER harbor N-glycans of the high-mannose type (Helenius and Aebi 2001). Thus, to determine the glycosylation status of the GP4D and GP5D proteins, we deglycosylated the TSP from transgenic plants using either

endo H or PNGase F, which have different substrate specificities; PNGase F removes almost all types of N-linked glycosylation, whereas Endo H removes only high-mannose N-glycans. Certain proteins having high-mannose N-glycans will be deglycosylated by both enzymes. Western blot analysis revealed that PNGase F- and endo H-treated GP4D and GP5D were detected at about 18 and 15 kDa, respectively, which corresponds exactly to their predicted size. However, the mYFP control showed no shift after treatment (Fig. 3). These results indicate that plant-made GP4D and GP5D proteins are glycosylated with ER-typical high-mannose residues.
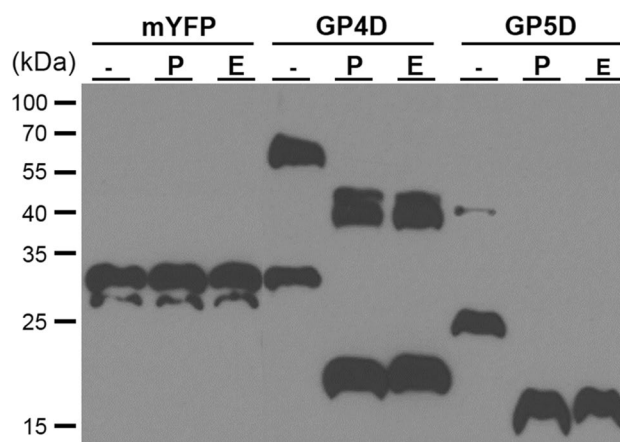

Fig. 3 Glycan analysis. Twenty micrograms of total soluble protein from transgenic *Arabidopsis* leaves expressing mYFP, GP4D, or GP5D protein was treated with endo H or PNGase F and analyzed by western blotting (under reducing conditions) with an anti-flag antibody. – no treatment, *P* PNGase F treatment, *E* endo H treatment
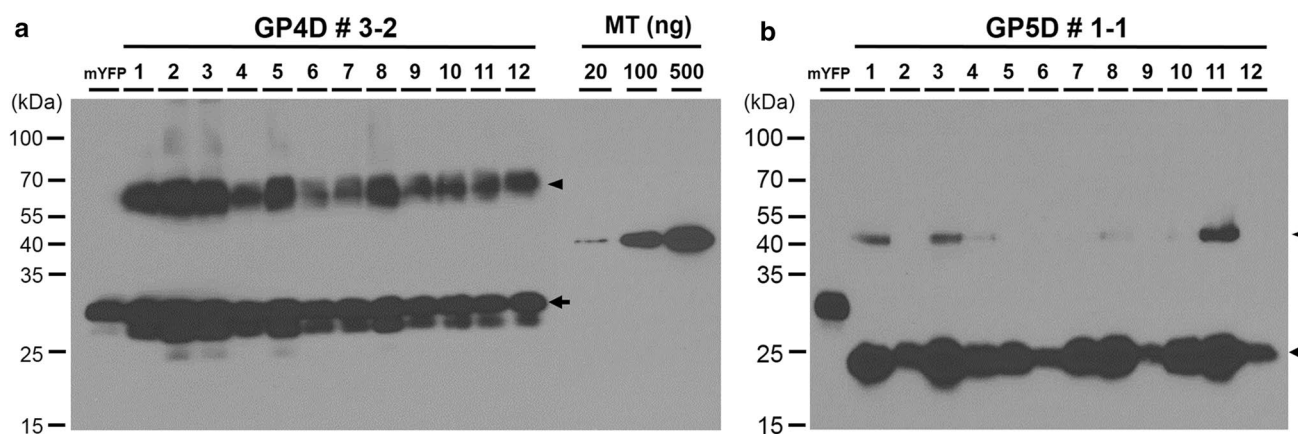

Fig. 2 Analysis of recombinant GP4D and GP5D protein expression in transgenic *Arabidopsis* plants (T2). Protein expression was analyzed in 12 plants expressing **a** GP4D and **b** GP5D proteins. Total soluble protein (30 μg) from extracts of transgenic plant leaves was separated on 12% SDS-PAGE gels, and expression of GP4D and GP5D was analyzed by western blotting with an anti-flag antibody. Arrows and arrow heads indicate monomeric and dimeric forms of each protein, respectively. *MT* multiple tag (40 kDa) from GenScript (M0101)

PRRSV-specific antibody responses and viral load in pigs

All pigs were then challenged with PRRSV JA142 strain 2 weeks after the last administration, and serum samples were collected and tested for IFA titer. At 49 and 56 dpt, the antibody titer after virus challenge was higher in pigs fed GP4D/GP5D transgenic plants than in pigs fed mYFP transgenic plants (Fig. 4a). Specifically, IFA antibody levels induced in the immunized group at 56 dpt were significantly higher than those in the control group ($p < 0.05$). These results show that vaccination with GP4D and GP5D proteins derived from transgenic *Arabidopsis* triggers a humoral immune response that generates virus-specific antibodies.

The viral load in serum and lung samples from pigs was analyzed by qRT-PCR (Fig. 4b, c). Pigs fed GP4D/GP5D transgenic plants showed lower mean peak virus titers than the pigs in the control group. The mean serum viral load in the immunized group fell from a high of $10^{4.82}$ TCID$_{50}$/ml at 49 dpt to $10^{3.2}$ TCID$_{50}$/ml at 56 dpt (Fig. 4b). In addition, the viral load in the lung was measured at 56 dpt to estimate viral clearance. Pigs fed GP4D/GP5D transgenic plants had a lower viral load in the lungs than pigs in the control group (Fig. 4c). One pig in the GP4D/GP5D transgenic plant group had a lung viral load of $10^{2.81}$ TCID$_{50}$/ml, lower than the mean peak lung virus titer of $10^{4.42}$ TCID$_{50}$/ml for this group. The mean virus titer in the lung of control pigs was relatively high at $10^{4.82}$ TCID$_{50}$/ml. Reductions in the viral load in serum and lung are likely due to PRRSV-specific antibody responses.

Cellular immune responses in pigs

The percentage of lymphoid immune cells was analyzed by flow cytometry (Fig. 5a, b). At 7 and 56 dpt, the percentage of Tregs (CD4$^+$CD25$^+$FoxP3$^+$) within the PBMC population was significantly lower in pigs fed GP4D/GP5D transgenic plants than in the control group (Fig. 5a). The total percentage of IFN-γ$^+$ lymphocytes in pigs fed GP4D/GP5D transgenic plants gradually increased, and was significantly higher than that in control pigs at 42 dpt (Fig. 5b). A similar trend was seen for cytotoxic T-lymphocytes (CD8$^+$IFN-γ$^+$) and Th1 cells (CD4$^+$IFN-γ$^+$), although the differences did not reach statistical significance (data not shown). These results indicate that viremia is kept in check and that plant-made GP4D and GP5D proteins confer a degree of cell-mediated immunity.

Pro-inflammatory cytokines in serum were evaluated by ELISA at 0, 42, and 56 dpt (Fig. 5c, d). At 56 dpt, the concentrations of TNF-α were significantly higher in pigs fed GP4D/GP5D transgenic plants than in control pigs (Fig. 5c). Similarly, after challenge, IL-12 levels were higher in pigs fed GP4D/GP5D transgenic plants (Fig. 5d). The simultaneous increase in TNF-α and IL-12 may be due to a reduction in the immunosuppressive effect of PRRSV, which is mediated by GP4D and GP5D antigens derived from the transgenic *Arabidopsis* leaves.

Clinical immune responses in pigs

To evaluate clinical responses against PRRSV, all pigs were euthanized at 56 dpt and gross and microscopic lung lesions were observed (Fig. 6). Microscopic lung lesion scores for pigs fed GP4D/GP5D transgenic plants were significantly lower than those for control pigs (Fig. 6a). The results of
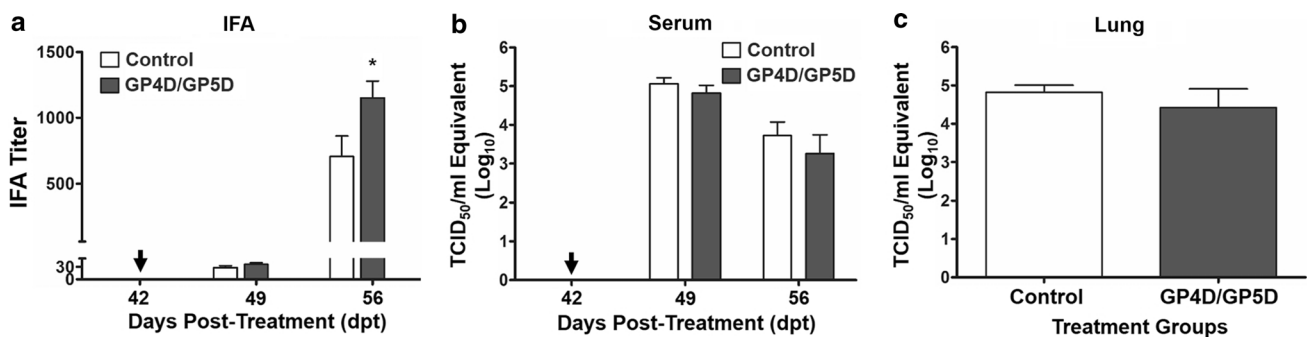

Fig. 4 Humoral immune responses in pigs orally immunized with transgenic *Arabidopsis* leaves, followed by PRRSV JA142 challenge. Five pigs per group were fed transgenic leaves expressing mYFP (control) or GP4D/GP5D protein (three times at 2 week intervals), followed by challenge with PRRSV JA142 strain at 42 dpt. **a** PRRSV-specific IFA titer in the serum of pigs at 42, 49, and 56 dpt. **b** Changes in the viral loads in pig serum at 42, 49, and 56 dpt. **c** Resid- ual virus load in the lung of pigs at 56 dpt, assessed by qRT-PCR. Virus titers were calculated based on a standard curve of the threshold cycle (C_t) number plotted against the known virus titer of JA142. Arrows indicate the time of challenge with PRRSV JA142 strain. *IFA* indirect immunofluorescence antibody, *TCID$_{50}$* 50% tissue culture infectious dose. Asterisks (*) denote values that are significantly different from each other ($p < 0.05$)

Fig. 5 Cellular immune response in pigs orally immunized with transgenic *Arabidopsis* leaves, followed by challenge with PRRSV JA142. **a** Regulatory T cells (CD4$^+$CD25$^+$Foxp3$^+$) and **b** total PBMCs expressing IFN-γ$^+$ were measured on 7, 14, 28, 42, and 56 dpt. **c** TNF-α and **d** IL-12 were analyzed by ELISA. *PBMCs* porcine peripheral blood mononuclear cells, *IFN* interferon, *TNF-α* tumor necrosis factor α, *IL-12* interleukin-12. Each bar represents the average percentage of each immune cell population in five pigs (± SEM). Asterisks (*) denote values that are significantly different from each other ($p < 0.05$)
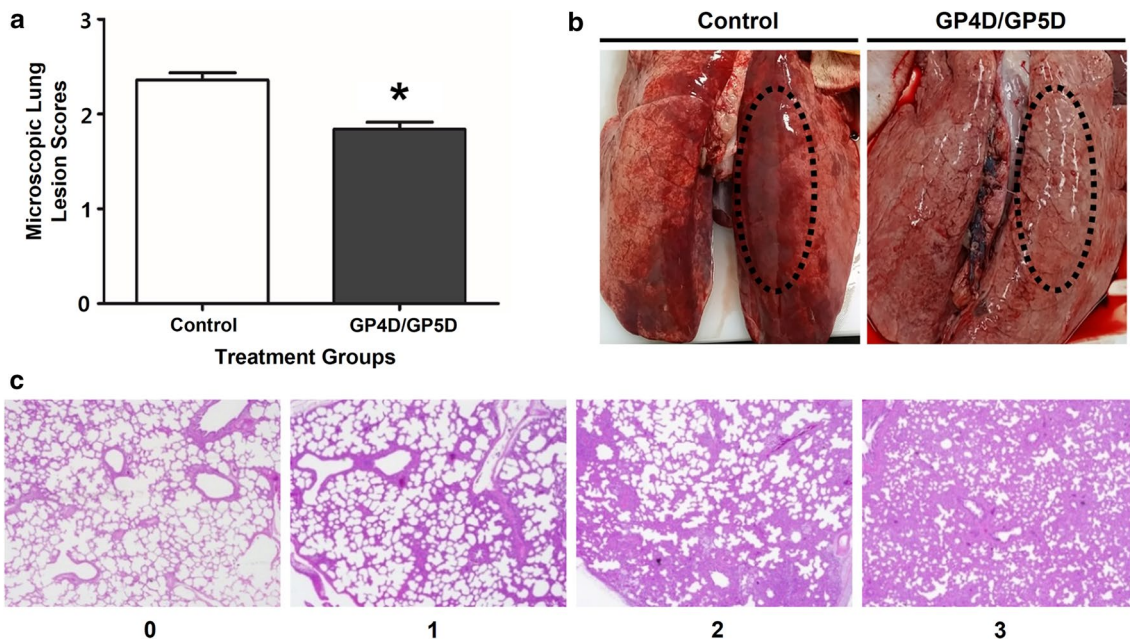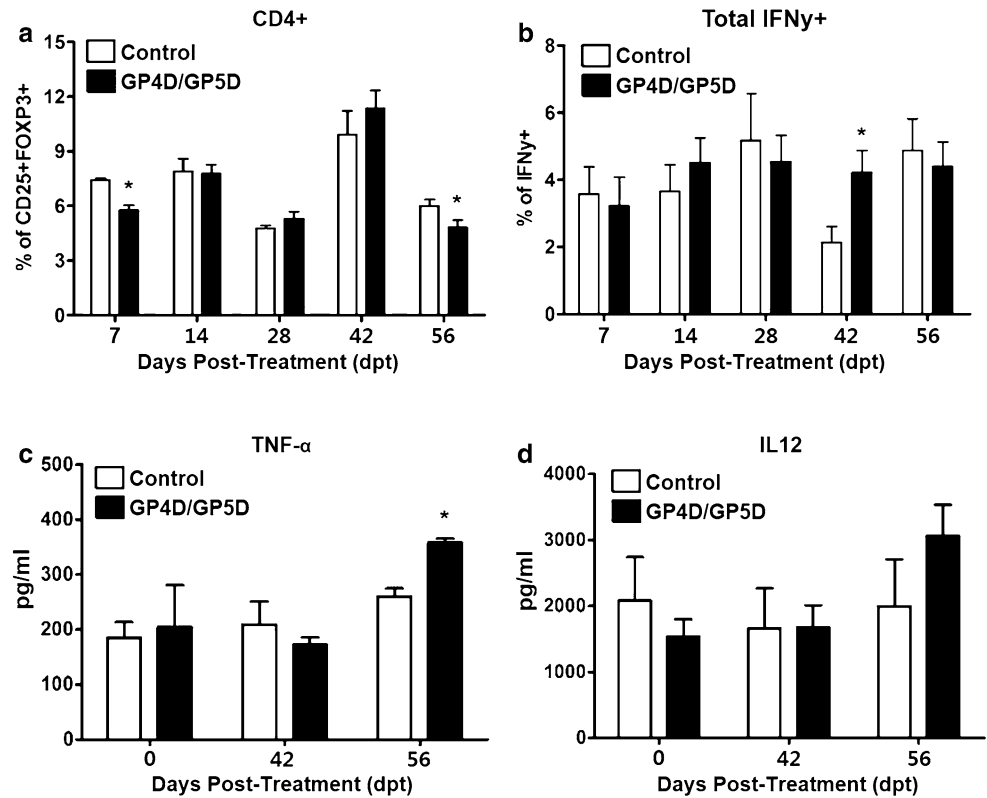

Fig. 6 Lower lung lesion scores in pigs orally immunized with transgenic *Arabidopsis* leaves, followed by challenge with PRRSV JA142. **a** Microscopic lung lesion scores and **b** gross lung appearance were examined after necropsy at 56 dpt. The dotted ellipses indicate the area of gross lesions. **c** Scoring of microscopic lung lesions was recorded on a scale of 0–3 as follows: 0, no lesion; 1, mild interstitial pneumonia; 2, moderate multifocal interstitial pneumonia; 3, severe interstitial pneumonia. Each bar represents the average microscopic lung lesion scores from five pigs (± SEM). Asterisks (*) denote values that are significantly different from each other ($p < 0.05$)

gross lung lesion analysis showed a trend similar to that observed for the microscopic lung lesion scores (Fig. 6b). As shown in Fig. 6b, the gross lung lesions in the GP4D/GP5D group were fewer in number and less red than those in the control group. The microscopic lung lesion scores ranged from 0 to 3 depending on the degree of inflammation in the lungs (Fig. 6c). The results demonstrate that anti-PRRSV responses were triggered by GP4D/GP5D transgenic plants. Average daily weight gain (ADWG) was calculated before and after challenge, and was similar in both groups (Fig. S3).

Discussion

Here, we report that immunization of pigs with plant-made antigenic GP4D and GP5D proteins induces immune responses against PRRSV. PRRSV glycoproteins GP4 and GP5 were selected as candidate antigens. GP5, a major envelope glycoprotein, is one of the key immunogenic proteins of PRRSV, which is used for the vaccine development (Kheyar et al. 2005; Pirzadeh and Dea 1998). GP4, a minor envelope glycoprotein that interacts with GP5 and contains a highly variable neutralizing epitope, is also a critical viral envelope protein (Costers et al. 2010; Das et al. 2010). Therefore, it is possible that a combination of GP4D and GP5D proteins might act synergistically to induce immune responses against PRRSV.

Plant-based vaccines against viruses that infect cattle and swine are safe and cost-effective, and provide both mucosal and systemic immunity (Buonaguro and Butler-Ransohoff 2010; Floss et al. 2007; Kang et al. 2005). In particular, plant cells protect the proteins due to their cell wall, which is advantageous for oral application (Sala et al. 2003). Thus, expressing proteins in plants has various advantages, although low yields are a major problem reported by various studies (Chan et al. 2013; Chen and Liu 2011; Chia et al. 2010; Hu et al. 2012; Vimolmangkang et al. 2012). To overcome this, codon bias, subcellular targeting strategies, and the use of untranslated regions have been studied (Gould et al. 2014; Habibi et al. 2017; Kim et al. 2014). Kim et al. (2014) suggested that the sequence upstream (−1 to −21) of the AUG initiation codon plays a pivotal role in translation efficiency. The previous studies report that the 5′-leader sequence (Ω) of TMV increases the expression and translation of the foreign gene (Gallie et al. 1987; Gallie 2002). Thus, to improve gene expression, we also used a plant expression vector system harboring the 5′ Ω leader sequence, which is incorporated upstream of the *GP4D* and *GP5D* sequences. In addition, protein translation might be inhibited by rare codons present in host plants. Gould et al. (2014) report that RNA processing and protein translation and folding are affected by codon bias when expressing synthetic genes in plants.

In general, proteins containing hydrophobic or membrane-associated domains are insoluble and difficult to recover. Rodriguez et al. (2001) reported that the expression of GP5 protein in *E. coli* increased when the TM was removed. Recently, Piron et al. (2014) reported high expression of truncated forms of GP4 [lacking the TM (GP4-Tm)] in transgenic *Arabidopsis* plants. Indeed, we failed to obtain any *Arabidopsis* plants harboring the full-length *GP4* and *GP5* genes showing detectable transgene expression (data not shown). Therefore, to improve the expression of GP4 and GP5 proteins in plants, we adopted several strategies, such as codon optimization, hydrophobic TM deletion, translation efficiency optimization (TMV Ω), and ER-targeting. Here, we found that GP4D and GP5D proteins comprised about 1.66% of total TSP derived from transgenic *Arabidopsis* leaf extracts. The previous studies reported unmodified GP5 levels of about 0.011 and 0.037% TSP in tobacco (Chia et al. 2010) and banana leaves (Chan et al. 2013), respectively.

Subcellular targeting to cellular compartments is also very important for high-level expression of target proteins (Fischer et al. 2004). Previously, plant-based vaccine proteins were targeted to the cytosol, ER, vacuoles, and chloroplasts to increase protein accumulation and improve stability (Habibi et al. 2017). We also investigated several subcellular targeting vector systems, including vacuoles, cytosol, chloroplasts, and ER, to improve the expression of transgenes in *Arabidopsis* (data not shown). Among them, we found that GP4D and GP5D were highly expressed using an ER-targeting vector system. The ER is an ideal organelle for increasing protein accumulation and stability, as well for controlling glycosylation (Aebi 2013). Nuttall et al. (2002) reported that the ER contains low levels of proteases and high concentrations of chaperones, which could assist recombinant proteins with respect to post-translational folding and stability. Currently, the ER-targeting system is widely used for high-level expression of recombinant proteins in plants because of these beneficial effects. Here, we developed an ER-targeting vector system containing the N-terminal VTS transit peptide and the C-terminal ER retention signal (KDEL) for plant transformation. We confirmed the exact localization of the mYFP protein in transgenic *Arabidopsis* plants by confocal microscopy analysis (data not shown). Furthermore, our glycan analysis suggested that GP4D and GP5D proteins were localized to the ER (Fig. 3).

In the present study, pigs were orally administered GP4D/GP5D transgenic plants leaves as a vaccine. None of the pigs showed any clinical adverse events, demonstrating that the vaccine is safe for oral administration. Serum and lung tissue samples from pigs fed GP4D/GP5D transgenic plants leaves showed lower levels of viremia and lower viral loads, respectively, than samples from control pigs. In addition, the microscopic lung lesion scores were significantly lower in the treated group than in the control.

These results are attributed to induced immunity against PRRSV due to expression of GP4D and GP5D proteins in transgenic leaves. Similar results were observed when pigs were fed transgenic tobacco leaves expressing GP5 protein (Chia et al. 2010) and transgenic banana leaves expressing GP5 protein (Chan et al. 2013). We found no significant difference in the ADWG of control and immunized animals, both before and after challenge with virus. These results are in agreement with studies in which animals were treated with different anti-PRRSV vaccines but showed no significant change in daily weight (Ellingson et al. 2010; Shabir et al. 2016; Wang et al. 2008).

Viral infection or vaccination elicits a humoral immune response in the form of virus-specific antibodies (Hu et al. 2012). Here, pigs administered GP4D/GP5D transgenic *Arabidopsis* leaves developed specific anti-PRRSV IgG antibodies. The antibody titer in GP4D/GP5D plants leaf-treated pigs at 49 and 56 dpt was higher than that in control pigs. Mice immunized with transgenic potato extract expressing PRRSV GP5 generated both serum and gut mucosal-specific antibodies (Chen and Liu 2011). A vaccination-dependent graded increase in the serum and saliva anti-PRRSV IgG and IgA antibody responses was observed in animals treated with transgenic banana leaves expressing GP5 protein, which is in agreement with our own results (Chan et al. 2013). We measured the levels of neutralizing antibodies, but detected none at significant levels. As the previous studies demonstrate (Scortti et al. 2007; Lee et al. 2014; Zuckermann et al. 2007), inactivated vaccines comprising PRRSV do not usually induce significant antibody responses by vaccination itself; however, vaccinated groups mount stronger immune responses after virus challenge than non-vaccinated groups due to the memory responses seeded by vaccination.

A robust cellular immune response plays a crucial role in controlling and suppressing infection (Amanna and Slifka 2011). Although cell-mediated immune responses elicited by PRRSV are unclear, the percentage of IFN-γ-secreting cells usually correlates with protection against PRRS outbreaks that cause reproductive failure in sows (Lowe et al. 2005). IFN-γ inhibits PRRSV replication by blocking RNA synthesis via dsRNA inducible protein kinase (Rowland et al. 2001). Here, we found that the percentage of IFN-γ-producing cells at 42 dpt was significantly higher in animals fed GP4D/GP5D transgenic plants. Moreover, significantly fewer Tregs were observed soon after administration of GP4D/GP5D transgenic plants and after challenge with virus, suggesting reduced production of these cells. Earlier studies show that PRRSV induces Tregs in pigs, and that this may play an important role in modulating immune responses against PRRSV by delaying the cellular immune response (Batista et al. 2004; Mateu and Diaz 2008; Silva-Campa et al. 2009). The increase in total IFN-γ$^+$ cells on the day of challenge, along with reduced production of Tregs, could

be a basis for keeping viremia in check, suggesting a cell-mediated immune response.

TNF-α, a major pro-inflammatory cytokine secreted by cells such as macrophages and activated T cells, has a pleiotropic function in promoting an antiviral state in uninfected neighboring cells; it also recruits lymphocytes to sites of infection (Huang et al. 2015). Here, we observed significantly higher induction of TNF-α after challenge of pigs fed GP4D/GP5D transgenic *Arabidopsis* leaves. Similarly, higher levels of IL-12 were measured in pigs fed GP4D/GP5D transgenic leaves. In agreement with the results obtained from the control group, the previous studies demonstrate the inhibitory effect of PRRSV on TNF-α production (Díaz et al. 2006; Gómez-Laguna et al. 2010). The simultaneous increase in the production of the TNF-α and IL-12 observed herein suggests that transgenic *Arabidopsis* leaves expressing GP4D and GP5D proteins inhibit the immunosuppressive effects of PRRSV. Increased induction of pro-inflammatory cytokines in the treated group may be associated with reduced viremia after challenge. Earlier studies demonstrate a role for TNF-α and other pro-inflammatory cytokines in inhibiting PRRSV replication (López-Fuertes et al. 2000; Shabir et al. 2016).

In this study, we used various approaches to increase the effectiveness of plant-based vaccines against PRRSV. There is a possibility that a combination of GP4D and GP5D proteins might synergistically induce immune responses against PRRSV. Das et al. (2010) reported that GP4 and GP5 proteins interact strongly, which could result in GP4 and GP5 proteins having increased protein stability. In addition, we are currently investigating GP3 protein expression in plants. It would also be interesting to examine the expression of recombinant proteins fused to the cholera toxin B subunit (CTB) with the view to using them as adjuvants or as a source of epitopes derived from GP3, GP4, and GP5 proteins.

In conclusion, we demonstrated successful overexpression of PRRSV-derived GP4D and GP5D antigenic proteins in *Arabidopsis*. Pigs immunized with transgenic plants expressing GP4D and GP5D proteins generated both humoral and cellular anti-PRRSV immune responses. The lower lung lesion scores and lower levels of viremia observed in immunized animals were considered to be direct outcomes of the PRRSV-specific antibody response and the production of pro-inflammatory cytokines elicited by the anamnestic response to antigenic PRRSV proteins. Thus, these findings demonstrate that plant-derived GP4D and GP5D vaccines provide an alternative approach to preventing PRRSV.

Author contribution statement CHA, W-IK and CYK: conceived and designed the experiments. CHA, SN and AK: performed the experiments. S-CP, YJJ, JHL, JCJ and Y-IP:

analyzed the data. S-JP and W-IK: contributed reagents/materials/analysis tools. CHA, SN, JCJ and CYK: wrote the paper.

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