Research Article

Use of virus vectors for the expression in plants of active full-length and single chain anti-coronavirus antibodies

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To extend the potential of antibodies and their derivatives to provide passive protection against enteric infections when supplied orally in crude plant extracts, we have expressed both a small immune protein (SIP) and a full-length antibody in plants using two different plant virus vectors based on potato virus X (PVX) and cowpea mosaic virus (CPMV). The αSIP molecule consisted of a single chain antibody (scFv) specific for the porcine coronavirus, transmissible gastroenteritis virus (TGEV) linked to the α-CH3 domain from human IgA. To express the full-length IgA, the individual light and heavy chains from the TGEV-specific mAb 6A.C3 were inserted into separate PVX constructs and plants were co-infected with both constructs. Western blot analysis revealed the efficient expression of both the SIP and IgA molecules. Analysis of crude plant extracts revealed that both the plant-expressed αSIP and IgA molecules could bind to and neutralize TGEV in tissue culture, indicating that active molecules were produced. Oral administration of crude extracts from antibody-expressing plant tissue to 2-day-old piglets showed that both the αSIP and full-length IgA molecules can provide *in vivo* protection against TGEV.

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1 Introduction

A potential method for protecting newborn animals against enteric pathogens is via the oral administration of neutralizing antibodies, or their derivatives. This approach, termed passive immunization, has been extensively investigated in the case of transmissible gastroenteritis coronavirus (TGEV). This virus infects both enteric and respiratory tissues, and causes close to 100% mortality in newborn pigs [1]. A mAb, 6A.C3, has been identified that is able to neutralize all TGEV isolates tested. This mAb recognizes a highly conserved epitope of the spike (S) globular protein that is probably essential for virus replication, since no neutralization escape mutants have been found [2]. A recombinant murine-porcine immunoglobulin A (IgA) isotype of mAb 6A.C3 produced in the milk of transgenic mice has been shown to be highly effective in the neutralization of TGEV when supplied orally [3, 4]. In addition, small immune proteins (SIPs; [5]), derived from mAb 6A.C3, expressed in mammalian cells

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Abbreviations: **CPMV**, cowpea mosaic virus; **dpi**, days post inoculation; **PVX**, potato virus X; **rIgA**, recombinant IgA; **SIP**, small immune protein; **TGEV**, transmissible gastroenteritis virus

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were able to efficiently neutralize TGEV in tissue culture and to protect against TGEV infections when supplied orally to newborn pigs (M. Bestagno *et al*., J. Gen. Virol., in press).

If passive immunization against TGEV, or other enteric pathogens, is to become a practical reality, it will be essential that large amounts of the appropriate antibody be produced at low cost. The production of biopharmaceuticals, such as vaccines or antibodies, by genetic engineering in plants has several advantages over methods based on bacterial or animal cell systems, including low cost, low risk of toxicity or contamination by animal pathogens, and the feasibility for large-scale production [6, 7]. Frequently, transgenic plants have been used to express relevant molecules for use in the formulation of experimental vaccines for humans and animals. Expression of recombinant antibodies (rAbs) has great potential to protect against viral infections and a wide variety of different rAbs has been successfully expressed in transgenic plants [6]. However, the use of transgenic plants for the expression of antibodies has nevertheless some disadvantages, such as the long time needed to obtain appropriate transgenic lines and the low yields of the expressed protein that are frequently encountered. In addition, the production of full-length antibodies depends on the simultaneous expression of the light (L) and heavy (H) chain peptides, which usually requires the time-consuming procedure of obtaining genetic crosses among the two types of transgenic plants.

An alternative approach for obtaining high levels of expression of foreign proteins in plants is to transiently express the gene of interest from a recombinant plant virus vector. Since viral genomes multiply within infected cells, this generally leads to the accumulation of high levels of the protein. Several plant viruses have been successfully engineered into vectors for the expression of foreign genes. We have previously reported the expression of a small immune protein (εSIP), consisting of a single-chain antibody (scFv) derived from mAb 6A.C3 fused to the CH4 domain of the S2 isoform of human IgE, in plants by means of cowpea mosaic virus (CPMV)- and potato virus X (PVX)-derived expression vectors [8]. This version of a SIP molecule was selected because the chains are able dimerize efficiently and stably via a cysteine residue at the C terminus of the CH4 domain of IgE S2. Plant extracts containing the SIP molecules were able to neutralize TGEV in tissue culture. Furthermore, oral administration of crude extracts from SIP-expressing plant tissue to 2-day-old piglets demonstrated that those extracts that showed the highest levels of *in vitro* neutralization could also provide *in vivo* protection against challenge with TGEV. However, the protection of the newborn animals was lower than that afforded by the full-length recombinant IgA, produced in mammalian cells, on which it was based. The potentially higher stability of the full-length secreted IgA antibody and derivatives based on it, especially on the mucosal surfaces, may be the reason behind the different behavior of the two types of antibody molecule. Alternatively, it may reflect the fact that the full-length mAb is able to mediate additional mechanisms of protection, such as antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC), which will not be invoked by the SIP molecule as it lacks the relevant portions of the constant region of the full-length antibody.

To extend the potential use of oral passive immunization, we report here the expression in plants, using viral vectors, of an anti-TGEV SIP molecule based on the CH3 domain of IgA (αSIP), which does not have a C-terminal cysteine, and the corresponding full-length recombinant IgA (rIgA). The performance of the different constructs *in vitro* and *in vivo* was assessed. The results obtained will allow the future optimization of methods for conferring passive immunity through the oral supply of plant extracts.

2 Materials and methods

2.1 Plasmids

Functional recombinant mAb 6A.C3 genes were originally described in [3]. The source of the sequence of the anti-TGEV αSIP for all the experiments was pcDNA-6AC3-huαSIP (Fig. 1a; Bestagno *et al*., J. Gen. Virol., in press). The source of the L and H chain recombinant sequences derived from mAb 6A.C3 were plasmids pINSLC6A and pINSHC6A (Fig. 1c), respectively [4]. The viral vectors used were pGR106 [9] and pBinP-NS-1 [10] for expression from PVX and CPMV RNA-2, respectively. Full-length CPMV RNA-1 was supplied by plasmid pBinPS1NT [11].

2.2 Construction of recombinant virus expressing anti-TGEV antibodies

To generate a CPMV-based vector containing the sequence of the anti-TGEV αSIP, pcDNA-6AC3-huαsip was modified by PCR-based mutagenesis. The sequence of an intron present in the original leader sequence was removed and a unique *Apa*I site at the 5′ terminus of the coding sequence was introduced, replacing the original ATG start codon. To achieve this, the forward primer ACTCTAGCCAAGCTTGTCGGGGCCCGGCTGGAG CCTGATCCTCCTGTTCCTCGTCGCTGTGGCTACAGG TGTGCACTCGGACATTGTGATGACCC was used. The reverse primer GCTAACCGAGAATTCTCAGAGTTCGT CGTGGTAGCAGGTGCCGTCCACC was used to fuse the sequence encoding an ER retention signal, HDEL, to the C terminus of the CH3 domain. The resulting construct (pYP-1), containing the modified version of the αSIP, was digested with *Apa*I and *Eco*RV and the 1.5-kb fragment encoding the sequence of the SIP was used to replace that

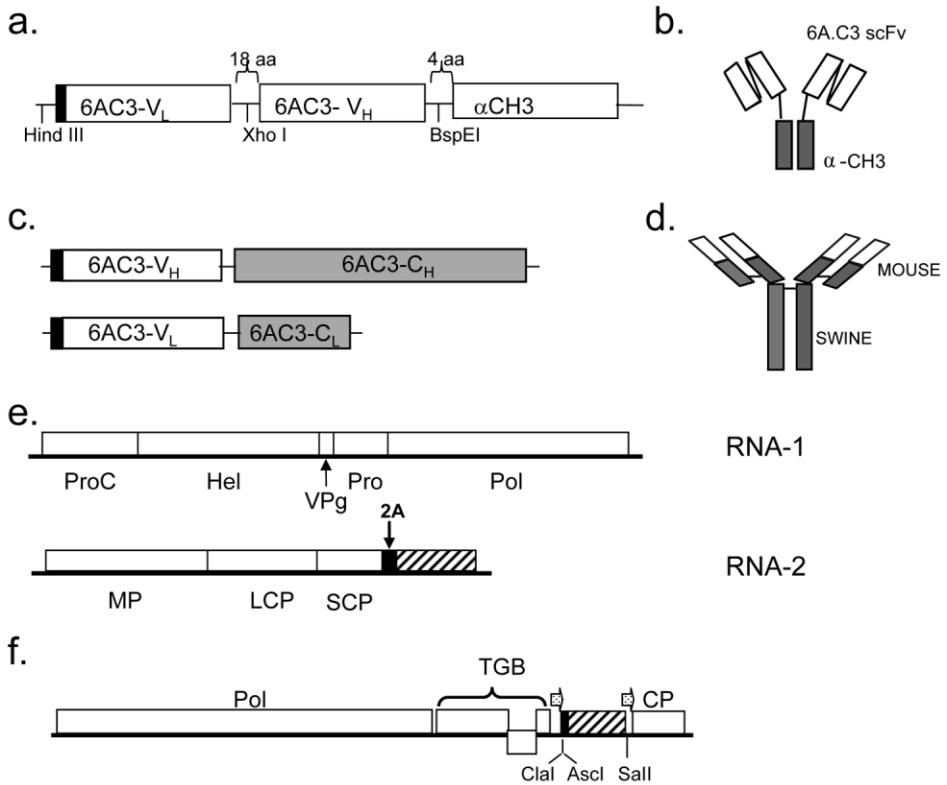

Figure 1. Constructs used to express αSIP and recombinant IgA in plants. (a) Schematic representation of the αSIP portion of plasmid pcDNA-6AC3-huαSIP with the regions derived from the variable domains of the light (V_L) and heavy (V_H) chains of mAb 6A.C3 and the CH3 domain from human myeloma IgA. The leader peptide is indicated by the black box. (b) Secondary structure of dimerized SIP with the regions derived from the 6A.C3 scFv and the CH3 domain shown as open and gray boxes, respectively. (c) Schematic representation of the heavy and light chains of rIgA derived from mAb 6A.C3. (d) Secondary structure of the assembled rIgA, with mouse- and swine-derived regions indicated as open and gray boxes, respectively. The leader peptide is indicated as a black box. (e) Structure of CPMV-huαSIP. (f) Schematic representation of PVX-huαSIP and PVX-rIgA. In (e) and (f) the sequence encoding the αSIP is indicated by the hatched box. The various virus-encoded proteins are indicated as: ProC, proteinase cofactor; Hel, helicase; Pro, proteinase; VPg, genome-linked viral protein; Pol, RNA-dependent RNA polymerase; MP, movement protein; LCP, large coat protein; SCP, small coat protein; TGB, triple-gene-block proteins; CP, coat protein.

of GFP in *Apa*I/*Stu*I-digested pBinP-NS-1 to give plasmid pBinP-YP1.

For cloning in pGR106, the αSIP gene in pcDNA-6AC3-huαsip was amplified by PCR, using CCATCGATCCATGGGCTGGAGC as the forward primer to produce αSIP constructs with its original murine leader peptide. A *Cla*I site (underlined) was introduced upstream of the SIP-specific sequence. As reverse primer GCGTCGACTCAGTAGCAGGTG containing a *Sal*I site (underlined), was used. For cloning of rIgA chains in pGR106, the sequences encoding the L and H chains of mAb 6A.C3 were amplified by PCR using the forward primers CCATCGATGGCGCGCCATGGAGTCACAGATTCAG, which includes an *Asc*I site (underlined), and CCATCGATCCATGAAATGCAGCTGGGTC, with a *Cla*I site (underlined), respectively. The reverse primers were: GCGTCGACTAAGCCTCACACTCATTC and GCGTCGACTATCCAACCAGGTATG for L and H chains, respectively. Both include an *Sal*I site (underlined). The PCR products were digested with *Cla*I and *Sal*I for the αSIP and H chain, and with *Asc*I and *Sal*I for the L chain, and ligated into *Cla*I/*Sal*I- or *Asc*I/*Sal*I-digested pGR106 to give pGR106-huαSIP, pGR106-LC and pGR106-HC. The structure of the αSIP and L and H chains constructs was verified by sequence analysis before appropriate *Agrobacterium* strains were transfected by electroporation. *A. tumefaciens* strain GV3010 was used for pGR106-antibody constructs and strain LBA4404 for pBinP-YP1.

2.3 Infection of plants

Plants were initially infected by agro-inoculation. *Nicotiana clevelandii* was used as the host for pGR106-huαSIP, pGR106-LC and pGR106-HC. *Agrobacterium* GV3101 cultures carrying pGR106 vectors were pelleted and resuspended to a OD_{600}=0.5 in a solution containing 150 μM acetosyringone, 10 mM MES, 10 mM $MgCl_2$, and incubated in this solution at room temperature for 2 h. Approximately 150 μL of the *Agrobacterium* suspension was applied with a syringe into the underside of three leaves

of young *N. clevelandii* plants. For the expression of rIgA, agrobacterium cultures carrying pGR106-LC and pGR106-HC were mixed (1:1) and used for co-inoculation of young *N. clevelandii* plants. In the case of pBinP-YP1 cowpea (*Vigna unguiculata)* plants were co-inoculated with bacteria containing pBinPS1NT, to provide RNA-1 for virus replication. Virus in crude extract of agroinoculated leaves was concentrated by precipitation with polyethylene glycol [12] and used for subsequent passaging.

2.4 Immunological detection of antibody expression in leaf tissue

Total protein was extracted from frozen powdered leaves using a buffer containing 50 mM Tris-acetate pH 8.0, 10 mM potassium acetate, and 1 mM EDTA. The extracts were filtered through Miracloth mesh and centrifuged to remove further debris. Protein samples were heat denatured in SDS sample buffer (30 mM Tris-HCl pH 6.8, 1.5% SDS, 10% glycerol, 0.1 mg/mL bromophenol blue) in the presence or absence of 2.5% 2-mercaptoethanol to achieve reducing or non-reducing conditions. Approximately 25 µg crude protein (as estimated by the Bradford assay) was separated on a 10% SDS-PAGE gel and electroblotted on to PVDF membrane (Immobilon P, Millipore). For detection of αSIP molecules, the membrane was probed with anti-human α-chain goat antibodies conjugated with peroxidase (KPL) diluted 1:5000 in PBS containing 0.1% Tween-20 (PBS/T). rIgA molecules were detected with goat anti-swine IgA antibodies (Nordic, Tilburg, The Netherlands), diluted 1:500 in PBS/T containing 0.3% BSA, and horseradish peroxidase (HRP)-conjugated rabbit anti-goat (Sigma) diluted 1:1000 in PBS as second antibody. Bound antibodies were detected by ECL using reagents supplied by Amersham Biosciences.

2.5 *In vitro* analysis of antibody activity

Infected plant tissue was collected at several times post inoculation, snap frozen in liquid nitrogen, ground to fine powder and crude plant extracts were prepared in 2 mL/g ice-cold 0.05 M sodium phosphate buffer pH 7.2. Cell debris was removed by centrifugation at high speed for 20 min, at 4°C. Supernatant containing total soluble plant proteins was used for the assays. The ability of αSIP and rIgA molecules in the plant extracts to bind to TGEV was determined by ELISA and virus neutralization assays, following previously reported procedures [13]. Purified TGEV virions were adsorbed to ELISA plates before the plant extracts (50 µL) were added to the wells. TGEV-bound αSIPs or rIgAs were detected with a goat anti-human IgA and goat anti-swine IgA antibodies, respectively (Nordic), diluted 1:500 in PBS/T containing 0.3% BSA, and HRP-conjugated rabbit anti-goat IgG (Sigma) diluted 1:1000 in PBS as the second antibody.

Neutralization assays were performed by combining 50 µL from a TGEV (PUR46-MAD) preparation of known titer with an equal volume of plant extract. At least three serial dilutions of the extract were analyzed in each assay. After incubation for 30 min at 37°C, the mixture was added to confluent monolayers of swine testis cells grown on 24-well tissue culture dishes. After adsorption of TGEV to cells for 45 min at 37°C, the medium was removed and replaced by an overlay medium and the cells incubated for a further 48 h at 37°C. The cell monolayer was then fixed and stained with crystal violet to visualize TGEV plaques. The neutralization index was calculated as the log of the ratio of the number of virus plaques in the absence of antibody to the number of virus plaques after the incubation with the antibody.

2.6 *In vivo* analysis of SIP activity

About 90–100 g of infected tissue was snap-frozen in liquid nitrogen, ground to fine powder, and extracted in 2 mL/g ice-cold 0.05 M sodium phosphate pH 7.2. After removal of cell debris, the cleared extract was frozen at –80°C and lyophilized. About 2.8 g lyophilized material were obtained from each sample. Then, 0.03 g of each sample was reconstituted with 1 mL phosphate buffer and the *in vitro* activity was assayed. To assess the ability of the plant expressed antibodies to confer *in vivo* protection, 0.3 g lyophilized plant tissue dissolved in 4 mL water were added to 6 mL milk. TGEV PUR46 C11 (10^7 PFU per animal) was added to the mixture and supplied to 2-day-old piglets via an oral canula. The piglets were subsequently fed, via baby bottles, with milk mixed with 0.3 g lyophilized plant tissue dissolved in 4 mL water. The antibody-containing mixture was administered twice more on the day of challenge and three times on the following 2 days post challenge. At 2 and 3 days post challenge, a time when virus levels reach a peak in TGEV-infected piglets [14], piglets were killed and virus titers in gut and lung tissue were determined as described by Jimenez *et al.* [15]. Protection against TGEV by plant extracts was expressed as the ratio of PFU obtained after the administration of extracts from plants infected with the wild-type vectors to those obtained when the corresponding SIP-expressing extracts were supplied. As positive controls, the protection afforded by the administration of 4 mL of a 1:10 dilution of ascitic fluid containing mAb 6A.C3 compared with ascitic fluid not containing the antibody and the protection afforded by 4 mL supernatants from Sp2/0 cells expressing the anti-TGEV αSIP compared with supernatants expressing a SIP of irrelevant specificity were assessed.

3 Results

3.1 Construction of recombinant viruses for the expression of anti-TGEV antibodies

The sequences encoding the L and H chains of the murine-porcine rIgA form of mAb 6A.C3 and the sequence encoding an αSIP based on a 6A.C3 scFv were inserted into two different plant virus-based expression systems. In the first instance, an expression vector based on the bipartite virus, CPMV, which had previously been used successfully to express high levels of a 6A.C3-derived εSIP [8] was used to express the equivalent αSIP. The second vector, based on the monopartite virus, PVX, was used to express both the full-length rIgA and the αSIP. To express αSIP using CPMV, the sequence encoding it (Fig. 1a, b) was inserted downstream of a foot-and-mouth disease virus 2A catalytic domain at the C terminus of the RNA-2-encoded CPMV polyprotein (Fig. 1e) to give plasmid pBinP-YP1. The 2A-mediated cleavage allows release of at least 90% of the αSIP protein with only one additional proline residue at its N terminus. The sequence of the αSIP was flanked by the leader peptide from the original 6A.C3 mAb at its N terminus. To optimize protein accumulation, an endoplasmic reticulum (ER) retention signal, the tetrapeptide HDEL, was added to the C terminus of the αSIP sequence, a strategy that had proved effective with the expression of equivalent εSIP using the CPMV system [8]. In the case of the PVX-based vector, the sequence of the anti-TGEV αSIP (Fig. 1a, b) was inserted into pGR106 (Fig. 1f), to give pGR106-huαSIP. The insertion positioned the αSIP sequence behind a duplicated coat protein sub-genomic promoter, allowing expression of free protein.

To express the full-length rIgA (Fig. 1c, d) the PVX-based system was chosen. Two constructs were generated, one expressing the L and the other the H chain. In both cases, the sequences were preceded by the leader peptide from the original murine 6A.C3 mAb, to allow the expressed peptides to be directed to the secretory pathway.

Infection with the modified viruses was initiated by agro-inoculation. *N. clevelandii* plants were used to propagate the PVX constructs, whereas cowpea was the host for the CPMV construct. For the expression of the full-length rIgA, a mixture of *Agrobacterium* cultures harboring the PVX-based plasmids pGR106-HC and pGR106-LC was used for inoculation.

3.2 Expression of huαSIP and full-length rIgA in plant tissue

Cowpea plants inoculated with sap from plants agro-inoculated with pBinP-YP1 in the presence of RNA-1 developed mild symptoms, the chlorotic spots being smaller in size and developing several days later than those resulting from a wild-type infection. The resulting virus was designated CPMV-huαSIP. RT-PCR of RNA extracted from these plants revealed that the SIP-specific insert was retained in the RNA-2 of CPMV-huαSIP (results not shown). Western blot analysis using anti-human α-chain antibodies of extracts from cowpea plants infected with CPMV-

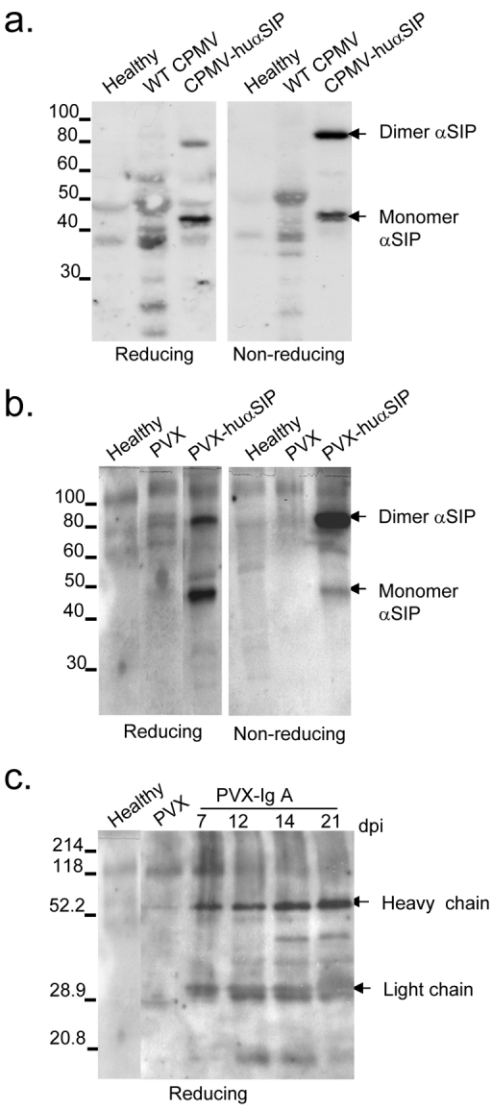

Figure 2. Western blot analysis of αSIP and rIgA expression in plants. (a) Extracts from either healthy cowpea plants or plants infected with wild-type CPMV (WT CPMV) or CPMV-huαSIP. (b) Extracts from either healthy *N. clevelandii* plants or plants infected with wild-type PVX or PVX-huαSIP. Membranes were probed with antibodies specific for human αSIP. The extracts were analyzed under reducing and non-reducing conditions, as indicated. The positions of the monomeric and dimeric forms of the αSIP are shown on the right and the sizes of the marker proteins are indicated on the left-hand side. (c) Extracts from healthy *N. clevelandii* plants, plants infected with wild-type PVX or with PVX-rIgA were probed with anti-swine IgA-specific antibodies. Samples were taken from systemically infected leaves at the indicated times, and analyzed under reducing conditions. The positions of the bands corresponding to the heavy and light chains are shown on the right and the sizes of the marker proteins are indicated on the left-hand side.

huαSIP revealed the presence protein of 43 kDa, corresponding to the monomeric form of the αSIP, when the protein was electrophoresed under reducing conditions (Fig. 2a). This band was not present when extracts of cowpea plants infected with wild-type CPMV were analyzed. In addition, a small amount of material corresponding to dimeric αSIP (approximately 85 kDa) was seen. Under non-reducing conditions, substantially increased amounts of the αSIP ran at 85 kDa, indicating that efficient dimerization of the expressed peptides had occurred *in planta* (Fig. 2a).

N. clevelandii plants agro-inoculated with PVX-based constructs developed symptoms at 7–9 days post inoculation (dpi) when plants were inoculated either with pGR106-huαSIP or with a mixture of pGR106-LC and pGR106-HC. The symptoms on plants expressing the antibody constructs were milder, and appeared at least 2 days later than those seen on plants infected with the empty PVX vector. The resulting virus infections were designated PVX-huαSIP and PVX-rIgA. RT-PCR of RNA extracted from infected plants confirmed retention of the inserted sequences, at least between 7 and 10 dpi (results not shown). Western blot analysis of crude plant extracts from leaves infected with the PVX-based constructs at 7 dpi revealed the presence of the αSIP as a band of the expected size (43 kDa.) when the sample was analyzed under reducing conditions. Under non-reducing conditions most of the protein ran as an approximately 85-kDa band, consistent with the formation of αSIP dimers (Fig. 2b).

Quantitative analysis by ELISA using antibodies against huαSIP showed that crude extracts from cowpea leaves contained levels of αSIP corresponding to about 0.2% of total soluble protein, whereas accumulation levels of αSIP of about 0.7% of total soluble protein were obtained in *N. clevelandii* plants using the PVX expression system.

Expression of the full-length rIgA was determined by Western blot analysis of crude extracts of *N. clevelandii* at different times post inoculation, using anti-swine IgA antibodies. Two bands of the expected sizes for the L and H IgA chains were observed in samples collected at 7–21 dpi (Fig. 2c). Although the amount of full-length antibody chains increased over time, several bands of smaller sizes, probably corresponding to IgA chain degradation products, were also detected in samples collected after 12 dpi. Therefore only samples collected at earlier times were used in subsequent experiments.

3.3 *In vitro* activity of αSIP and rIgA produced in plants

To analyze whether the 6A.C3-derived αSIP and recombinant IgA molecules produced in plants have the capacity to bind to TGEV, ELISA assays were carried out using plates coated with partially purified TGEV particles. Extracts from cowpea plants infected with CPMV expressing αSIP showed a low level of binding that was only slightly above that obtained from equivalent extracts from leaves infected with the wild-type virus (Fig. 3a). However, the binding obtained with extracts from *N. clevelandii* leaves infected with PVX-huαSIP was significantly higher than that of the negative control (wild-type PVX), and similar to that obtained with human αSIP expressed in mammalian cells (Fig. 3a). Levels of binding significantly above the negative control were also obtained with extracts from plants infected with PVX-rIgA. The binding activity shown by these extracts was only slightly lower than that of mAb 6A.C3 produced in mammalian cells which was used as a positive control (Fig. 3a), indicating

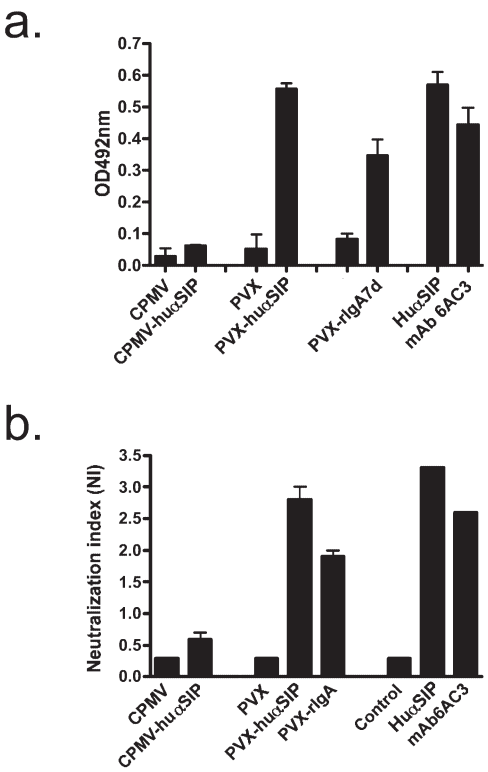

Figure 3. Activity of plant-expressed αSIP and full-length rIgA molecules. (a) ELISA assay of the binding of plant-expressed antibodies to partially purified TGEV particles. Sap extracts from cowpea plants infected with CPMV-huαSIP and *N. clevelandii* plants infected with the PVX-huαSIP or PVX-rIgA were analyzed. In each case, extracts from plants infected with the corresponding wild-type virus (PVX or CPMV) was analyzed as a negative control, and the binding activity of the αSIP molecule expressed in mammalian cells (huαSIP), and the parental mAb 6A.C3 were used as positive controls. (b) TGEV neutralization assay of antibodies produced in plants. Plants infected with the appropriate wild-type virus were used as negative controls for each plant sample. The neutralization index was calculated as the log of ratio of virus plaques in the absence of antibody/virus plaques after the incubation with the antibody. The results obtained with the plant extracts were compared with those obtained with the parental antibody (mAb 6A.C3) and the αSIP (huαSIP) expressed in mammalian cells. The negative control (control) was an SIP of irrelevant specificity expressed in mammalian cells. Both the neutralization and ELISA data are the mean of at least two independent experiments.

that the assembly of the L and H chains into active immunoglobulin molecules is effective in the PVX-infected plants.

To further confirm the activity of recombinant antibodies expressed in plants, TGEV neutralization assays were performed. The activities of the plant extracts were compared with the neutralization obtained with the equivalent constructs expressed in mammalian cells (positive controls), as well as with supernatants from mammalian cells expressing a SIP molecule of irrelevant specificity (negative control). The results of the neutralization assays mirrored those obtained in the ELISA assays. As expected, negligible levels of neutralization were obtained when extracts from plants infected with the wild-type viruses or the unrelated SIP were used (Fig. 3b). Low levels of neutralization, compared with the positive control, but above those found in extracts from plants infected with wild-type CPMV, were found in cowpea leaves infected with CPMV-huαSIP (Fig. 3b). In contrast, neutralization values similar to those obtained with positive controls were found with extracts from *N. clevelandii* leaves expressing αSIP molecules using the PVX vector. Similarly, high levels of neutralization were obtained in extracts of leaves infected with PVX-rIAg (Fig. 3b).

These results confirmed that both the αSIP and the rIgA molecules produced in plants are able to bind and to neutralize TGEV particles *in vitro*. The lower levels of neutralization obtained with the extracts from leaves infected with CPMV-huαSIP, compared with extracts from leaves infected with PVX-huαSIP or PVX-rIgA, are consistent with the relative levels of antibody expression as determined by Western blot analysis and ELISA.

3.4 *In vivo* protection of newborn animals

The ability of plant extracts containing anti-TGEV αSIP or rIgA to protect animals against TGEV challenge was assayed using the samples showing the highest binding and neutralization levels (Fig. 3). Extracts were prepared from the upper leaves of *N. clevelandii* plants systemically infected with PVX-huαSIP or PVX-rIgA as well as from plants infected with the parental PVX vector. Extracts were orally supplied as lyophilized material, mixed with the milk used to feed newborn pigs. The binding and neutralization activity of the lyophilized material was reconfirmed before administration of the samples. Generally, the lyophilized samples showed activity that was about 70% relative to that found in fresh crude extracts (results not shown). This partial loss of activity, probably resulting from repeated freeze-thaw treatments was similar for all the samples, and the lyophilized samples were used for the *in vivo* assay.

Previous experiments showed that administration of supernatants from mammalian cells expressing anti-TGEV antibody molecules (used in this work as positive controls) simultaneously with a TGEV challenge gives results similar to those obtained when the antibody was supplied prior to the challenge (M. Bestagno *et al.*, J. Gen. Virol., in press). Therefore, TGEV challenge was carried out simultaneously with the first administration of the antibody-containing or control samples. Extracts were further administered over a 2-day period. After that, TGEV levels were determined in gut and lung of the challenged animals, and protection was expressed as a ratio of the virus titer obtained with antibody-containing extract, relative to the equivalent extract without the antibody (Fig. 4). The results showed that supplying pigs orally with extracts from *N. clevelandii* plants expressing αSIP or full-length rIgA caused a notable reduction in virus titers compared with extracts from leaves infected with wild-type virus. Plant extracts expressing αSIP reduced TGEV titers by over 100-fold in the gut and more than 10 000-fold in the lung of the infected animals. The reduction of virus titers was lower in the gut but higher in the lung compared with that achieved with the supernatants from mammalian cells containing the αSIP (Fig. 4a). By contrast, plant extracts containing full-length rIgA greatly reduced virus titers in the gut of challenged animals, but were almost ineffective in lungs (Fig. 4b). The decrease in virus titer with the rIgA-containing plant extracts was less than that found with the parental mAb 6A.C3, particularly in the lung. This difference is consistent with the lower binding and neutralization activity of the plant-expressed antibody found in the *in vitro* assays (Fig. 3b). Thus, *in vitro* assays can provide a good estimation of the actual capacity of plant extracts to confer *in vivo* protection in target animals.

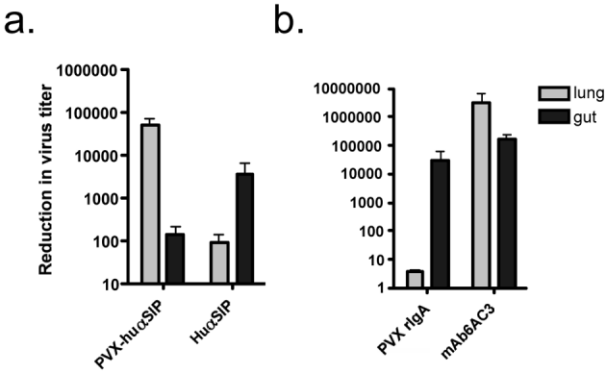

Figure 4. *In vivo* protection of newborn pigs against TGEV as determined by the reduction in virus titers. The virus titers in the lung and gut were determined after administration of plant extracts to piglets that were challenged with TGEV. (a) Virus titers after administration of plant extracts expressing αSIP. Positive controls consisted of αSIP expressed in mammalian cells. (b) Virus titers after administration of rIgA-expressing plant extracts. Positive controls consisted of parental antibody 6A.C3 expressed in mammalian cells. The reduction in virus titer was determined as described in the Materials and methods. Mean values and SDs correspond to the reduction in virus titers in three to four piglets tested for each antibody.

4 Discussion

The results presented here demonstrate that plant-expressed antibody molecules based on IgA molecules are able to neutralize TGEV infections both *in vitro* and *in vivo*. These observations confirm and extend the results previously reported using SIP molecules based on the CH4 domain of IgE (εSIP; [8]). The latter molecules were selected for initial study because they are able dimerize efficiently and stably via a cysteine residue at the C terminus of the CH4 domain, and it was believed that this could be important in ensuring their activity when expressed in plants. The CH3 domain of the αSIP contains a cysteine as its penultimate C-terminal amino acid but in the parental IgA molecule this is involved in binding to the J-chain rather than participating in antibody dimerization. However, recent results with αSIP molecules expressed in mammalian cells showed that in the absence of the J chain, this cysteine can also promote efficient dimerization (M. Bestagno *et al*., J. Gen. Virol., in press). The fact that dimeric αSIP molecules were detected in Western blots under non-reducing conditions (Fig. 2a, b) and neutralization was obtained with plant extracts containing the αSIP molecules indicates that this dimerization also occurs efficiently in plants.

There is a striking difference between which viral vector is most efficient for the expression of α and εSIPs. With the εSIP, expression from CPMV resulted in approximately 20 times more protein accumulation than was found when a PVX vector was used [8]. This difference was ascribed to presence of an HDEL sequence, allowing ER retention, on the SIP expressed from CPMV, a sequence absent from the PVX-expressed protein. Thus, the PVX-expressed SIP is expected to be secreted to the apoplast. By contrast, in the current report, higher levels of expression were obtained with PVX compared with CPMV. Although the precise reasons for this difference have not been investigated in detail, they probably reflect a combination of factors. For example, the relatively high levels of accumulation of αSIP expressed from PVX may reflect the greater stability of an SIP based on a secretory antibody (IgA) in the relatively hostile environment of the apoplast compared with that based on IgE. Alternatively, the difference may reflect some aspect of how well the various heterologous sequences are tolerated by the different virus vectors. Whatever the precise cause, the difference in expression levels indicates that optimization of expression strategy and targeting is likely to be required to achieve maximum accumulation levels of individual antibody constructs.

A particularly significant aspect of the current work is the demonstration that a fully functional IgA can be produced in plants using virus vectors expressing the individual L and H chains of an mAb. There has only been one other report of the assembly of a full-length antibody using this approach employing Tobacco mosaic virus rather than PVX [16]. Although the authors of that work reported that the antibodies could bind their cognate antigen, indicating correct assembly, no data about their *in vivo* activity were presented. In contrast, our data demonstrate that the IgA molecules expressed from PVX not only bind to antigen but are also active in neutralization both *in vitro* and *in vivo*.

In terms of the comparative efficiency of neutralization of SIPs and full-length antibodies, the *in vivo* data on the reduction of virus titers in the gut provide some support for the idea that full-length antibodies are more efficient at the primary site of infection. However, the plant-expressed SIP molecule appears to be considerably more effective than the full-length antibody at reducing the virus titer in the lungs. This may be a consequence of the smaller size of the SIP molecules allowing more efficient release from the crude plant extract so that it can penetrate the animal tissue more readily. It may also reflect the generally greater tissue penetration found with SIP molecules [17], although this might also be expected to occur when SIP molecules expressed in mammalian cells are administered, which was not observed in our experiments (Fig. 4). One possible explanation is that other materials within the plant extracts have a protective-adjuvant role but we have no direct evidence to support this conclusion.

Overall, the results presented here confirm and extend our previous conclusion that plant-expressed antibodies are efficacious molecules that can provide immediate protection against virus infections, as required in new-born animals or healthcare workers. The fact that full-length neutralizing antibodies, as well as smaller derivatives such as SIPs, can be expressed via viral vectors will extend the range of uses to which the technology can be applied. This points the way to a new approach to controlling such diseases as SARS-CoV and other enteric and respiratory pathogens.

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