**ORIGINAL PAPER** 



## Transient Expression in Cytoplasm and Apoplast of Rotavirus VP6 Protein Fused to Anti-DEC205 Antibody in *Nicotiana benthamiana* and *Nicotiana sylvestris*

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#### Abstract

Rotavirus is the most common cause of severe diarrhea in infants and children worldwide and is responsible for about 215,000 deaths annually. Over 85% of these deaths originate in low-income/developing countries in Asia and Africa. Therefore, it is necessary to explore the development of vaccines that avoid the use of "living" viruses and furthermore, vaccines that have viral antigens capable of generating powerful heterotypic responses. Our strategy is based on the expression of the fusion of the anti-DEC205 single-chain variable fragment (scFv) coupled by an OLLAS tag to a viral protein (VP6) of Rotavirus in *Nicotiana* plants. It was possible to express transiently in *N. benthamiana* and *N. sylvestris* a recombinant protein consisting of the single chain variable fragment linked by an OLLAS tag to the VP6 protein. The presence of the recombinant protein, which had a molecular weight of approximately 75 kDa, was confirmed by immunodetection, in both plant species and in both cellular compartments (cytoplasm and apoplast) where it was expressed. In addition, the recombinant protein was modeled, and it was observed that some epitopes of interest are exposed on the surface, which could favor their immunogenic response.

Keywords Nicotiana · VP6 · DEC205 · Transient expression · GFP

## Introduction

The most important cause of severe childhood gastroenteritis in the world is Rotavirus (RV). The genus RV belongs to the family Reoviridae, they are non-enveloped viruses that are approximately 100 nm in diameter. The virion is composed of three layers of proteins that encompass the viral genome, which consists of eleven segments of double-stranded RNA (dsRNA) and codes for six structural proteins (VP1 to VP4, VP6 and VP7) and six non-structural ones [1, 2]. The inner layer of the virion is formed by 60 dimers of the VP2 protein and small amounts of RNA polymerase (VP1) and guanyltransferase (VP3), which together form the core of the virus. Around the core is a second intermediate layer composed of

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<sup>1</sup> Departamento de Ingeniería Genética, Centro de Investigación y de Estudios Avanzados del IPN, 36824 Irapuato, Guanajuato, Mexico 260 trimers of the most abundant structural protein of the virion (VP6) forming a double-layer particle. The outermost layer is made up of two VP7 and VP4 proteins. The smooth surface of the virus is composed of 780 copies of the VP7 gly-coprotein organized in the form of trimers, while 60 spicules formed by VP4 project outward from the viral surface [3, 4].

To date, two attenuated RV vaccines have been authorized to be applied worldwide (Rotarix, GSK and Rotateq, Merck). Rotarix is composed of a human RV strain and RotaTeq is composed of five human-bovine recombinant RV strains [5, 6]. However, the protection induced by these vaccines against gastroenteritis was 80% in high- and middle-income countries, while it was only 39% and 70% in some low-income countries in Africa and Asia [5, 6]. For these reasons, it is necessary to study new platforms for the expression and production of recombinant RV proteins that can serve as vaccines based on "non-living" viruses that induce broadly heterotypic protection based on highly conserved antigens. On the other hand, with the application of modern biotechnology and the development of genetic transformation, the necessary foundations were laid for the use of plants as safe, effective, and affordable alternative systems to produce a wide variety of recombinant proteins of pharmaceutical interest, enzymes, antibodies, and hormones [7, 8]. Recombinant proteins can be produced in plants by stable genetic transformation or transient expression. Proteins generated by transient expression (magnifection) have been shown to have benefits, as human pathogens are not involved, sterility is not required during production, expression is rapid and high level for use in clinical trials for Food and Drug Administration (FDA) approval [9, 10]. However, no system is totally optimal for the expression of any recombinant protein in plants, due to the physical characteristics and the therapeutic application of each product [7].

Some research groups have worked to obtain transgenic plants that express some proteins that make up the RV capsid, with the aim of developing a possible oral or nasal vaccine in a plant system. The VP6 protein was expressed in Nicotiana benthamiana leaves in potato virus X (PVX) fused to the virus capsid or free. The VP6 produced in plants was mixed in vitro with the VP2 protein produced in baculovirus, both proteins were assembled into virus-like icosahedral particles (VLP) [11]. The assembly of VP6 in VLP suggests that possible post-translational modifications in the plant do not affect the ability of the protein to assemble. In addition, the production of specific antibodies against VP6 was observed after the first 15 days of the first inoculation in mice with total protein extracts expressing the antigen, indicating that the protein retains the property of being highly immunogenic by the parenteral route [12]. In another study, VP7 and VP6 were expressed in potato and it was shown that VP6 retained the property of forming trimers such as the native protein [13]. On the other hand, VP6 has been expressed with other proteins that are part of the capsid of the virus in tomatoes and tobacco [11-18]. All this background shows that plants can produce recombinant antigens that retain their immunogenic epitopes [15, 19, 20].

In this work, the transient expression of a scFv antibody coupled by an OLLAS tag to the VP6 protein in two different plant expression systems in two cell compartments was proposed. The OLLAS tag has been used successfully as an internal sequence for the fusion of monoclonal antibodies and antigens, improving the immunodetection of the recombinant proteins [21]. In general, this strategy has the purpose that in the future the antigen expressed in plants is used and that it is capable of directing the antigen to the dendritic cells to generate a faster and more powerful immune response, in addition to generating a safe production system and economic.

#### **Materials and Methods**

#### **Expression Vectors**

The coding sequence for VP6 was obtained from the National Center for Biotechnology Information (NCBI)

database (GenBank: U65988.1). Optimization of the VP6 gene sequence was performed. For this, the use of codons from Murine rotavirus EDIM and Nicotiana benthamiana (http://www.kazusa.or.jp/codon/) was done. The optimized sequence of VP6 was synthesized (GenScript, USA). Subsequently, the sequence of the VP6 gene was amplified with the binding sites to the scFv-anti-DEC205-OLLAS gene (KpnI) and the pICH31070 vector (BsaI) using the following primers: Forward: 5'-GGTACCATGGATGTG CTGTACTCTATCTCACGTAC-3' and the reverse: 5'-TTT GGTCTCAAAGCTTCACTTTACCAGCATGCTTC-3'. The scFv-anti-DEC205-OLLAS sequence was obtained by PCR amplification from a work previously reported by our research group [20] (GenBank ID: KC596074). The following primers were used to obtain the sequence: Forward: 5'-TTTGGTCTCAAGGTATGGGATGGTCTTGCA TCATTC-3' with the Bsal site and Reverse: 5'-GGTACC GCCCATCAACCTAGGTCCCAATTCATTCG-3' with the KpnI site for ligation with the pICH31070 vector and the VP6 gene, respectively. The VP6 sequence was fused to the scFv-anti-DEC205 by OLLAS tag consisting of 10 amino acids (GFNAELGPRL) reported by Park and cols. [21], which facilitates the identification of recombinant proteins (Fig. 1). This sequence was first cloned in pBlue-Script SK + vector (Stratagene, La Jolla, CA, USA) for subsequent cloning into the expression vector.

The amplification conditions of the VP6 and scFvanti-DEC205-OLLAS gene were an initial denaturation at 98 °C for 2 min, denaturation at 98 °C for 30 s, annealing at 69 °C for 30 s, and extension at 72 °C for 30 s. The reaction was carried out for 30 amplification cycles using the Platinum SuperFi II DNA Polymerase enzyme (Thermo Fisher) following the manufacturer's instructions. The products were analyzed in 0.8% agarose gels (Sigma-Aldrich) (Fig. 2A). The expression vector for plants of deconstructed virus pICH31070-scFv-anti-DEC205-OLLAS-VP6 was constructed by restriction ligation. The resulting plasmid was used to transform E. coli (DH5 $\alpha$ ), using the method described by Sharma and Schmike [22]; the transformation was performed with an Easyject Optima electroporator (Equibio, England). Recombinant clones were analyzed by restriction enzymes and confirmed by sequencing. Vectors were introduced into the Agrobacterium tumefaciens (GV3101) by electroporation. For the expression of the recombinant proteins in the cytoplasm and apoplast, the vector pICH15879 [23] and pICH20155 [24] were used, respectively; these vectors were supplemented with the vectors pICH31070-scFv-anti-DEC205-OLLAS-VP6 or pICH7410-GFP and the integrase module (pICH14011) [24] and untransformed Nicotiana plants were used as negative controls [10].



Fig. 1 Schematic representation of the vector construction and expression of the recombinant proteins

## **Plant Material and Growth Conditions**

A volume of 200 µL of seeds was taken, these were washed with 1 N sulfuric acid for 90 s, then washed 3 times with sterile deionized water for 45 s, followed by a 10% bleach wash (1 mL) for 9 min with intervals of 3 min with stirring and 3 min at rest, it was washed again three times with deionized water for 45 s, followed by another 10% bleach wash for 6 min with stirring and resting, finally washed again three times with water and seeded in petri dishes containing MS medium with vitamins (MS Vitamins 4.43 g/L, sucrose 30 g/L, phytagel 2.7 g/L and kanamycin 150 mg/L). Petri dishes were incubated at 28 °C with a photoperiod of 16 light hours and 8 dark hours for one week. Subsequently, 6 to 8 seedlings were placed in jars with MS Vitamins medium where they were incubated for 2 weeks under the conditions already mentioned; once they turned two weeks each plant was passed to the ground for acclimatization and three weeks they were incubated with the same conditions of light, darkness, and temperature, and if necessary fertilizer was added. 5 weeks after germination, the plants were used for agroinfiltration. Three biological replicates were made with 12 plants for the expression in each cell compartment of both plant species.

## Agrobacterium tumefaciens-Mediated Nicotiana Plant Magnifection

The A. tumefaciens strains carrying the deconstructed virus vectors were incubated at 28 °C for 48 h in YEB/ Rif/Cb or YEB/Rif/Km liquid medium (meat extract 5 g/L, yeast extract 1 g/L, peptone 5 g/L, sucrose 5 g/L, MgSO<sub>4</sub> 0.1 g/L, pH 7.0, rifampicin 50 mg/L and kanamycin 50 mg/L or carbenicillin 100 mg/L) until reaching an optical density (OD<sub>600nm</sub>) of 1.5, once the optical density was obtained, a mixture of A. tumefaciens was prepared containing the vectors (pICH31070 with the scFv-anti-DEC205-OLLAS-VP6 gene, pICH20155, pICH15879, pICH14011 and pICH-GFP) necessary for the transformation of the plants. For the transformation of the plants with the gene, a mixture was prepared with equal volumes of the A. tumefaciens strains with the scFv-anti-DEC205-OLLAS-VP6 gene contained in pICH31070, pICH20155, or pICH15879 and pICH14011; for the expression of the positive control, the strains with the pICH20155 or pICH15879, pICH14011, and pICH-GFP vectors were mixed. Acetosyringone (200 mM) was added and the mixtures were incubated for two hours at room temperature. The mixtures were centrifuged at 5500 rpm for 15 min, the obtained pellets were resuspended in the infiltration buffer (10 mM 2-N-morpholino ethanesulfonic acid (MES) pH 5.5, 10 mM MgSO<sub>4</sub>) to obtain a final dilution of 1:10 and have a  $DO_{600nm}$  of 0.15. The infection process of both Nicotiana benthamiana and Nicotiana sylvestris plants was carried out by wounding the underside of the leaf and the bacterial suspension was injected with the needleless syringe. Once infected, the plants were incubated at 25 °C with a photoperiod of 16 light hours and 8 dark hours for 10 to 12 days. Once the days of incubation were completed, the leaf tissue was harvested and macerated in mortar with liquid nitrogen and lyophilized for later use [10, 20, 25, 26].

#### **Total Soluble Protein Extraction (TSP)**

For the extraction of total soluble protein, the Tris–HCl buffer pH 7.2 (20 mM Tris–HCl, 10% sucrose, 2 mM EDTA, 150 mM m-bisulfite sodium, 1 mM PMSF, 2% PVPP) was used in a proportion 1:1 (w/v). The plant tissue was

homogenized with the extraction buffer with the help of the vortex and incubated for 10 min at room temperature. Subsequently, it was centrifuged at 12,000 rpm for 15 min at 4 °C and the supernatant was recovered, the last step was repeated until the supernatant was left without traces of plant tissue [26–29].

#### **SDS-PAGE and Western Blot analysis**

The amount of total soluble protein (TSP) was quantified by the method of Bradford using bovine serum albumin as standard. The integrity of the extracts was verified by separating the proteins by SDS-PAGE electrophoresis and staining the gels with Coomassie blue, for this 40 µg of protein was denatured at 95 °C for 5 min and loaded into polyacrylamide gels (SDS-PAGE) at 10%. They were subsequently electro-transferred to a polyvinylidene difloride membrane (PVDF Hybond-P+) (Amersham Pharmacia Biotech) in semi-dry equipment (Trans-Blot® SD Semi-Dry Transfer Cell BIO-RAD, USA), using transfer buffer with methanol (Tris 3.03 g/L, Glycine 14.4 g/L, methanol 20%), under a potential of 12 V for 45 min. The membrane was subsequently blocked with a 5% milk solution (Skim Milk, Difco) in 0.01% 1X-Tween-20 PBS for 1 h at room temperature under constant stirring. After blocking, the membranes were washed 5 times with 0.01% 1X-Tween-20 PBS vigorously, followed by incubation in the corresponding primary antibody for 1 h at 37 °C and overnight at 4 °C with constant stirring. For the detection of the recombinant protein, two different primary antibodies were used, due to the availability of each antibody; for the detection of the protein with the OLLAS tag, the supernatant of a rat anti-OLLAS monoclonal antibody was used in a 1:10 dilution in blocking solution. On the other hand, the goat anti-VP6 antibody of rotavirus capsid (Santa Cruz Biotechnology, Inc) was used in a 1:3000 dilution in blocking solution. After incubating with the primary antibody, the membrane was washed 5 times with 0.01% 1X-Tween-20 PBS vigorously. Subsequently, the membranes were incubated 1 h at room temperature under constant agitation with the secondary antibody, in this case the goat anti-rat (Invitrogen) and the rabbit anti-goat (Invitrogen) antibody, both coupled to horseradish peroxidase (HRP) in a 1:3000 and 1:5000 dilution, respectively. The membranes were again washed 5 times with 0.01% 1X-Tween-20 PBS vigorously and then three washes were given under constant agitation for 10 min. Finally, the specific binding with the chemiluminescent substrate ECL Thermo Scientific was detected, putting the membranes in contact with photographic plates, and then revealed [15, 20].

#### **Recombinant Protein Modeling**

The nucleotide sequence of the gene was translated into its amino acid sequence to subsequently obtain the quaternary structure of the protein; for this, the website http://expasy. org/tools/dna.html was used, once the amino acid sequence was obtained, it was introduced to the Protein Homology/ analogY Recognition Engine V2.0 (Phyre 2) program to search homologies with other reported proteins and obtain a PDB format of the recombinant protein [30, 31]. Finally, the sequence in PDB format was introduced in the PyMOL program for modeling. In addition, some epitopes of interest for protein detection were searched in the structure, as well as some domains and epitopes involved in rotavirus protection.

## Results

## Obtaining the scFv-anti-DEC205-OLLAS-VP6 Recombinant Gene

The sequence coding for the scFv-anti-DEC205-OLLAS-VP6 gene contained in pICH31070 plasmid was successfully constructed. To corroborate the correct insertion of the genes, a restriction analysis of the cloned fragment was made in the pBluescriptSK (+) cloning vector and in the pICH31070 expression vector (Fig. 2B and C) and sequenced. Restriction analysis and sequence confirmed the presence of the inserts in the open reading frame. The Blast of the results obtained from the sequencing showed 100% identity with the synthetic sequence scFv-DEC205-OLLAS reported by Coconi-Linares et al. [20] and 98% identity with the VP6 coding sequence reported by Chu and Greenberg [32].

# Transient Expression in Cytoplasm and Apoplast of *N. benthamiana* and *N. sylvestris*

In order to evaluate and compare the transient expression system of heterologous proteins mediated by deconstructed viral vectors and confirm that it is being carried correctly, the expression of the green fluorescent protein (GFP) was observed during the beginning, course, and end of the experiment in *N. benthamiana* and *N. sylvestris* in both cell compartments of expression: cytoplasm and apoplast (Fig. 3). In *N. sylvestris*, expression of GFP was observed from the third day in the cytoplasm with a maximum of visible expression on the tenth day and in apoplast it was observed from the fifth day with a maximum of expression until the tenth day Fig. 2 A Agarose gel showing the amplification product of the VP6 gene (Lane 1) and the scFv anti-DEC205-OLLAS gene (Lane 2). **B** Vector pBluescriptSK (+) containing the scFv-anti-DEC205-OLLAS-VP6 gene digested with *BsaI*. **C** pICH31070 vector containing the scFv-anti-DEC205-OLLAS-VP6 gene (Lane 1) digested with *EcoRV* (Lane 2) and with *KpnI* (Lane 3). MW: Lambda DNA/EcoRI plus HindIII Marker

**Fig. 3** Comparison of GFP expression in *N. sylvestris* and *N. benthamiana* from the first day of infection to the tenth day of incubation in the two cell expression compartments: cytoplasm and apoplast. The leaves were examined with ultraviolet light



Day/Cell	Cytoplasm		Apoplast	
compartment/Study	N. Sylvestris	N. benthamiana	N. Sylvestris	N. benthamiana
model.				
1				
3	( )			
5				
7				
9				
10			C C	

(Fig. 3). In *N. benthamiana*, GFP expression was observed from the second day in cytoplasm, having a maximum of visible expression on the tenth day and in apoplast it was observed from the third day with a maximum level of visible expression on the tenth day (Fig. 3). No phenotypic changes were observed in agro-infiltrated plants with respect to control plants.

#### **Analysis of Protein Expression**

Once the total soluble protein was extracted from the agroinfiltrated plants with the Tris–HCl buffer at pH 7.2, their integrity was evaluated by 10% SDS-PAGE electrophoresis with 40  $\mu$ g of sample (Fig. 4). In Fig. 4 the overexpression of GFP in the cytoplasm of both species of agro-infiltrated plants is observed. On the other hand, the presence of the recombinant protein composed of the scFv-anti-DEC205-OLLAS fused to the VP6 protein was determined by Western Blot in the two cell compartments (cytoplasm and apoplast) of the two plant species. For the detection of the protein, the primary antibody rat anti-OLLAS (Fig. 5A, B) and goat anti-VP6 (Fig. 5C) of RV were used as already mentioned above. Using these antibodies, a molecular weight band of about 75 kDa was detected that correspond to the fusion of the antigen-antibody. As can be seen, it was possible to detect the recombinant protein in both plant species (N. benthamiana and N. sylvestris) both in cytoplasm and in apoplast (Fig. 5A-C). In Fig. 5A, 40 µg of total protein was used as mentioned in M&M and it was possible to detect the recombinant protein expressed in apoplast in N. benthamiana. As the recombinant protein was not detected in the cytoplasm with 40 µg, for Fig. 5B and C, 60 µg of total protein was used, and the recombinant protein was identified in the cytoplasm in N. benthamiana and in both compartments in N. sylvestris, giving a stronger signal in apoplast than in cytoplasm in N. sylvestris.



**Fig. 4** Analysis of the total soluble protein extracts of *N. sylvestris*, *N. benthamiana*, and of the control samples with the Tris–HCl pH 7.2 extraction buffer. The box indicates the expression of the GFP protein. MW: BenchMark<sup>TM</sup> Protein Ladder



NB: N. benthamiana. NS: N. sylvestris. C-: Negative control. IB: Infection buffer. GFP: Green Fluorescent Protein. A: Apoplast. C: Cytoplasm. RP: Recombinant protein. C+: Positive Control.

Fig. 5 Detection of the scFv-anti-DEC205-OLLAS-VP6 recombinant protein (RP, Mw 75 kDa) by Western Blot. MW: BenchMark Pre-Stained Protein Ladder. A Detection of C+ (scFv anti-DEC205-OLLAS, Mw 30 kDa) and RP in apoplast expressed in *N. benthami*- *ana.* **B** Detection of C+ and RP in cytoplasm expressed in *N. benthamiana*. **C** Detection of RP in apoplast and cytoplasm expressed in *N. sylvestris* 

#### **3D Structure of the Recombinant Protein**

The scFv-anti-DEC205-OLLAS-VP6 recombinant protein was designed by fusing the variable regions of the heavy and light chain of the antibody against the DEC205 receptor both linked by an adapter of (Gly<sub>4</sub>Ser)<sub>3</sub> and the antigen (VP6) by the OLLAS tag. With the PDB format obtained from the Phyre2 program, protein modeling was performed using the PyMOL program. As can be seen (Fig. 6A) in the modeling obtained, the recombinant protein consists mostly of  $\beta$  sheets, in the case of the VP6 protein it consists of  $\alpha$  helices and  $\beta$  sheets highlighting in two different colors ( $\beta$  sheets in magenta color and the  $\alpha$  helices in blue color). In addition, the fusion regions between the heavy chain variable region and the light chain variable region of the antibody are highlighted, in this case the adapter (Gly<sub>4</sub>Ser)<sub>3</sub> (green color), and the fusion between the scFvanti-DEC205 antibody and the VP6 protein through the OLLAS tag (yellow color) (Fig. 6B). Finally, one of the most important domains and epitopes of the recombinant protein stands out: the OLLAS tag and the domain of residue 332-397 of the VP6 protein (red color), the OLLAS tag and the domain of residue 332-397 are exposed on the surface of the protein, in the case of the VP6 protein the domain is of great importance since it has been reported

Fig. 6 Three-dimensional structure of the scFv-anti-DEC205-OLLAS-VP6 recombinant protein. A The secondary structure of the recombinant protein is shown indicating the  $\alpha$  helices and the  $\beta$  sheets in different colors, both of the antibody and the antigen. B Secondary structure of the protein indicating the adapters in different colors: in green the adapter of (Gly<sub>4</sub>Ser)<sub>3</sub>, and in yellow the OLLAS tag. In orange and red the VP6, the red color in the VP6 indicates a residue 332-397 of the VP6 protein. The blue color indicates the variable region of the antibody heavy chain against DEC205, the magenta color shows the variable fragment of the antibody light chain. C and D Globular structure of the recombinant protein indicating two epitopes of interest for the detection of the protein, as

well as for the induction of an

immune response

to be one of the domains that can confer greater protection against rotavirus (Fig. 6C and D).

### Discussion

To date, the FDA has approved two rotavirus vaccines in 2005 and 2008; however, these showed nearly identical rates of intussusception. On the other hand, there is evidence to suggest that rotavirus disease may be an independent risk factor for intussusception. Therefore, the WHO emphasized that the benefit-risk profile of both licensed RV vaccines remains favorable, and the benefits outweigh the risk of intussusception; however, further investigation is needed on the risk of intussusception intestinal attributable to RV disease [33, 34]. A large group of researchers are currently focusing on generating alternatives for vaccine production; however, there are some limitations that impede their progress. Among the main limitations are the production platforms, the specificity for antigen delivery, the adequate stimulation of the immune response, the yield of the recombinant proteins, and, above all, the high production costs. An alternative to overcome some of these limitations is plants [27]. The production of rotavirus capsid proteins in plants has shown great interest, since different working groups have focused on the production of one or more proteins that



make up the capsid of the virus. On the other hand, to overcome the limitation of specificity for antigen delivery, new vaccination strategies are being developed by directing the antigens specifically to dendritic cells, through the use of specific antibodies against a dendritic cell receptor coupled to the antigens, which will allow the processing and delivery of antigens by means of MHC I and MHC II to T cells [35].

In this work, *N. benthamiana* and *N. sylvestris* were transiently transformed with the construction of the scFv-anti-DEC205-OLLAS-VP6. So far, reports of VP6 production in transgenic plants are focused on the production of the VP6 protein as such or co-expressed with other proteins that are part of the virus capsid [11–18, 35]. However, none of these studies have focused on the expression in plants of the antigen (VP6) directed toward dendritic cells using scFvanti-DEC205. Binding of the antigen to an antibody directed toward a cellular receptor has been shown to improve immunogenicity and induce T cells that generate broad antibodymediated responses, as demonstrated in the work by Badillo-Godinez et al. [5], where  $\alpha$ -DEC205-VP6 induced up to a 45% IgA-independent protection.

VP6 is the most immunogenic protein and is a potential target for vaccine development, this constitutes the first work on the expression of the VP6 protein directed toward dendritic cells by binding to an antibody in plants [18]. Using Nicotiana spp. as a model, N. benthamiana and N. sylvestris were transiently transformed with the construct that codes for recombinant protein expression. So far there are only three reports of expression in Nicotiana spp., one of them is the expression of VP6 [11], co-expression of VP2 and VP6 [36], and the another is the expression of the scFv-anti-DEC205 [20]. However, to date there are no reports on the expression of rotavirus proteins (VP6) in N. sylvestris. In addition, both species of Nicotiana were transformed into two cell compartments: cytoplasm and apoplast. By analyzing the expression patterns by Western blot of the recombinant protein obtained from agro-infiltrated plants, it was possible to detect, in the two Nicotiana species (N. benthamiana and N. sylvestris), the recombinant protein of an expected size of 75 kDa (Fig. 5).

The use of a smaller amount of total soluble protein for the identification of the recombinant protein in apoplast than in cytoplasm in this work is consistent with that reported by the Kavanagh group; they expressed nanobodies in cytoplasm, chloroplast, and apoplast of *N. benthamiana* and found that the recombinant protein accumulates at an exceptionally high level in apoplast (up to 30% of foliar protein) compared to cytoplasm and chloroplast [26]. We could not make such an accurate calculation of the final concentration because it lacked purified protein. As for the expression of the GFP recombinant protein, the same did not happen, as can be seen in Fig. 3, there was greater expression in the cytoplasm than in apoplast. In addition, comparing the expression in both species of plants, it can also be observed that in *N. benthamiana* 

is faster in terms of expression time of GFP in apoplast and cytoplasm and that there is a greater intensity of fluorescence, which indicates that there is a greater production of GFP (Fig. 3). It is necessary to highlight that despite observing GFP in the agro-infiltrated leaves of both *N. benthamiana* and *N. sylvestris* in the two cell compartments (cytoplasm and apoplast), only a 26.9 kDa band could be observed in the lanes corresponding to the extraction of GFP from the cytoplasm (Fig. 4). This may be due to different factors; however, there are reports where it was observed that the type of leaves is an effective factor for the level of production and processing of the recombinant protein [37].

As already mentioned, it appears that N. benthamiana leaves are more effective for the expression of the GFP in both cell compartments (apoplast and cytoplasm) compared to N. sylvestris (Fig. 3). This is consistent with what was reported by Hashemi [37], where they expressed transiently the human growth hormone in potatoes, tobacco, and lettuce. However, they found that in potatoes and tobacco, there was greater expression compared to lettuce leaves. Although the expression levels in tobacco and potato leaves were really high, tobacco produces high levels of toxic alkaloids and phenolic substances that are released during the milling and extraction of proteins and that can interfere with protein performance recombinant. Therefore, although GFP could be detected in apoplast, it is possible that a considerable amount of recombinant protein is degrading and therefore cannot be visualized by Coomassie staining. The same could be happening with the extraction of the scFv-anti-DEC205-OLLAS-VP6 protein [26].

Modeling of the scFv-anti-DEC205 fused by the OLLAS tag to the VP6 protein was performed (Fig. 6). The result of the model gives us an approximation of what aspect the recombinant protein could acquire and if true, the fusion of the viral protein with the anti-DEC205 would not alter the ability of the antibody to acquire a suitable conformation for the recognition of the DEC205 receptor. In addition, in modeling, it can be seen that the OLLAS tag is exposed on the surface of the protein, which is ideal for the detection of the recombinant protein (Fig. 6D). On the other hand, it is reported that one of the epitopes or domains that induce greater protection against rotavirus is residue 332–397 of the VP6 protein [38]. By selecting this residue in modeling, it can be seen that this epitope is exposed on the surface of the viral protein (Fig. 6D), which indicates that the recombinant protein may be able to generate a potent immune response through two pathways, one of which is through the introduction of the antigen to dendritic cells by scFv-anti-DEC205 and generate a cellular immune response by activating T lymphocytes mediated by the presentation of antigens by the major histocompatibility complex. On the other hand, being VP6 one of the subunits of the most immunogenic rotavirus capsid and presenting one of the domains that induce greater protection against rotavirus on its surface, a humoral and/or cellular immune response can be generated only from the recognition of the viral or epitope protein.

On the other hand, in this work, the sequence of the recombinant protein was optimized; these codon optimization strategies are based on the change of nucleotides within the gene sequence to increase the expression of recombinant proteins [39]. However, despite using this codon optimization process, no significant increase in the expression of the recombinant protein was observed on SDS-PAGE. Codon bias between the transgene and host expression is known to affect the expression levels of heterologous proteins [40]. At present, for the expression of recombinant proteins, the impact of codon optimization remains quite unpredictable, as there are multiple interrelated factors (promoter strength, A + T content, mRNA secondary structure, mRNA stability) that affect protein expression by optimizing a genetic sequence [39-41]. Therefore, based on all the current information on codon optimization, it is difficult to predict which strategy, if any, will design the "optimal" gene sequence, so it would be necessary to make different versions of the gene to determine the best one version to be expressed in the expression model [41].

In conclusion, according to the results obtained in this work and what has been reported by other work groups, we consider that the fusion of the scFv-anti-DEC205-OLLAS-VP6 could be an alternative to combat gastroenteritis caused by rotavirus and that its transient expression in the apoplast in *N. benthamiana* is an alternative to eliminate many of the limitations that currently exist for the production of a vaccine such as production platforms, specificity, adequate stimulation of the immune response, the risk of carrying antigens of pathogens, the performance of recombinant proteins, and, above all, the high production costs. However, there are still many more experiments to get to this point. In addition, we to improve and optimize our expression platform and protein purification, as well as the codon optimization of the sequence.

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Author Contributions JFC-E: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing—original draft, and Writing—review & editing. MAG-L: Conceptualization, Methodology, Validation, Resources, Writing—review & editing, Supervision, Project administration, and Funding acquisition.

#### Declarations

**Conflict of interest** The authors declare that they have no conflict of interest.

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