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RESEARCH

Production of Human Rotavirus and *Salmonella* Antigens in Plants and Elicitation of fljB-Specific Humoral Responses in Mice

Louis-Philippe Bergeron-Sandoval · Aurélie Girard · François Ouellet · Denis Archambault · Fathey Sarhan

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Abstract A *Nicotiana benthamiana* transient expression system was used to express single antigen and dimeric combinations of the human rotavirus (HRV) VP7 and a truncated VP4 (VP4Δ) proteins fused with *Salmonella typhimurium*'s flagellin fljB subunit. Immunoblot analyses using rabbit antibodies generated against these proteins demonstrated that the constructs were successfully expressed with yields ranging from 0.85 to 31.97 μg of recombinant protein per gram of fresh leaf tissue. Expressing the single and dimeric antigens has no effect on plant growth and development except for VP7 and VP4Δ::VP7, which show mild necrotic lesions. Immunization of mice with proteins from leaves transformed with constructs bearing the fljB moiety elicited an fljB-specific humoral response. The *Nicotiana benthamiana* transient system is efficient to express multiple combinations of pathogen proteins and demonstrates the potential of generating a *Salmonella typhimurium* subunit vaccine in plants.

Keywords Enteric pathogens · Flagellin · *Nicotiana benthamiana* · Plant-based vaccines · Transient expression

Introduction

The World Health Organization (WHO) estimates the death toll from diarrheal diseases at nearly 1.9 million deaths per year. Young children are at particular risk, especially in developing countries. Furthermore, untreated early childhood diarrheal diseases were found to impair growth, physical and cognitive development, which may later decrease human potential and productivity [1]. The most common enteric pathogens are adenoviruses, astroviruses, human caliciviruses, rotaviruses, *Campylobacter jejuni*, toxigenic *Escherichia coli*, *Salmonellas*, *Shigellas*, and *Vibrio cholerae*. The wide diversity of bacterial and viral enteric pathogens that cause diarrhea and gastroenteritis is a serious challenge for proper etiological diagnostic and development of treatments [2]. Epidemiological surveys on enteric pathogens in various regions of the world confirmed that 70% of diarrheal diseases are foodborne [3].

Salmonella, a causal agent of gastroenteritis in humans, is one of the most common bacterial foodborne pathogen [4]. The WHO Global Salm-Surv public health databank monitors the distribution of *Salmonella* serotypes and outbreaks worldwide. The *Salmonella typhimurium* serovar, the second most frequent strain found in humans [4], is a host-generalist that infects a wide range of animals. It is the major cause of food poisoning resulting from consumption of infected meat or poultry. The current vaccine, Poulvac ST (Fort Dodge Animal Health Inc, Overland Park, KS)®, is used to reduce *Salmonella enteritidis* infection in chickens. The use of a vaccine generated from disarmed live pathogens presents some risks like reversion to virulence or hypersensitive immune responses to vaccine components. A *Salmonella typhimurium* subunit vaccine would thus represent a safe alternative. Flagellin, a component of bacterial flagella, has been identified as a pathogen-associated molecular pattern (PAMP) that is recognized by the defense system in all eukaryotes. *Salmonella* expresses two flagellin proteins, fliC and fljB, known as strong immunological

L.-P. Bergeron-Sandoval · A. Girard · F. Ouellet · D. Archambault · F. Sarhan (✉)
Département des Sciences Biologiques, Université du Québec à Montréal, CP 8888 Succ. Centre-ville, Montreal, QC H3C 3P8, Canada
e-mail: sarhan.fathey@uqam.ca

elicitors [5]. Generation of subunit vaccines against these proteins could thus be of particular interest for epidemic salmonellosis disease control.

The Global Alliance on Vaccines and Immunizations (GAVI) identified a rotavirus vaccine as one of the top three priority vaccines for international development [6]. The human rotavirus (HRV) is a highly infectious agent causing infections that range from asymptomatic to severe dehydrating gastroenteritis, often resulting in hospitalization and sometimes death. Transmission of the virus is mostly fecal-oral but some studies have reported evidence for possible spread via the respiratory tract [7]. HRV is a triple layered icosahedral particle with two surface structural proteins that determine the serotypes. The G type is determined by the antigenic diversity of the VP7 glycoprotein and the P type is determined by the diversity of the VP4 protein [8]. The HRV peculiar structure causes a complex epidemiological pattern. This is exacerbated by the fact that temporal and geographical fluctuations allow multiple strains to circulate simultaneously in a given population [9]. The infectivity of HRV is dependent on the trypsin-like treatment of the viral particle. In the gastrointestinal tract, the conformation of the flexible minor outer capsid VP4 is altered by proteolytic cleavage and high pH, which results into polypeptides VP8* and VP5*. These subunits facilitate early virus–cell interactions, cell binding, entry, hemagglutination, and neutralization [10, 11]. The major outer capsid protein VP7 also contains epitopes for virus attachment to the host cell membrane and decapsidation process [12]. Therefore, VP4 and VP7 were chosen by many investigators as candidate antigens for subunit vaccine development. Current mono- and multivalent vaccines against HRV (RotaTeq by Merck Frosst and ROTARIX by GlaxoSmithKline) are very expensive, and thus are not available to the majority of third world populations [13]. Problems associated with the use of these vaccines are hypersensitivity to their components and the possible induction of intussusception or intestinal malformations [14]. In addition, they are not recommended for immunocompromised infants, infants with pre-existing chronic gastrointestinal conditions, and infants beyond 14 weeks of age at the time of first immunization [15]. The availability of a safe, effective, and affordable rotavirus vaccine would thus represent a global public health breakthrough.

Plants have been increasingly explored for the production of biomedicines and vaccine components. The edible plant-based vaccine technology offers an alternative to the traditional approaches used for immunization, particularly in the developing world where storing and administering classical vaccines are often major problems. Vaccines delivered orally increase safety measures by eliminating the need for needles. In addition, biosafety concerns are eliminated since plant pathogens do not replicate in animals and humans. Plants are thus an attractive production platform for recombinant vaccines because of their rapid engineering, low cost, high safety, and scalability [16]. Also of interest is the fact that edible vaccines target mucosae, which is essential to achieve stimulation of both the systemic and the mucosal immune networks. Mucosal immune responses represent a first line of defense against most enteric pathogens, as they are essential to block the bacteria or virus in the gut prior to epithelial cell infection [17].

In this report, we describe the expression of multiple combinations of rotavirus and *Salmonella* fljB flagellin proteins in a *Nicotiana benthamiana* transient expression system. The purified fljB proteins from plant extracts elicit an fljB-specific humoral response in mice, demonstrating the potential of generating a *Salmonella typhimurium* subunit vaccine in plants.

Materials and Methods

Amplification of the Native VP7, VP4, and fljB Genes

MA104 cells were maintained in Dulbecco minimal essential medium (DMEM) with 5% (v/v) fetal bovine serum. For cell infection, the human rotavirus Wa strain of the G1P1A[8] serogroup was incubated for 30 min at 37°C in DMEM supplemented with 10 μg/ml porcine pancreatic trypsin (Sigma-Aldrich), then confluent 2-day old MA104 cultures were inoculated with the activated viruses. The viral titers were determined and calculated as the median tissue culture infective dose (TCID50) per milliliter [18]. The infected cells were further incubated in serum-free DMEM containing 5 μg/ml porcine pancreatic trypsin until 90% of the cells exhibited a cytopathic effect. After three freeze/thaw cycles, the cell culture supernatant was collected by centrifugation for 15 min at 3,000×*g* at 4°C. The rotavirus genomic RNA was extracted from the supernatant using TRIzol® Reagent (Invitrogen) and was reverse-transcribed to complementary DNA (cDNA) using random hexadeoxyribonucleotides (pd(N)6; GE Healthcare), as previously described [19]. The cDNAs corresponding to VP7Δ (amino acids 84–332) and VP4Δ (amino acids 1–336) were then amplified by PCR using specific primers (Table 1) for cloning in various vectors. Genomic DNA from the *Salmonella enterica* serovar *Typhimurium* SL1344 strain was extracted by alkaline lysis of the bacterial culture, and the fljB gene was amplified by PCR using specific primers (Table 1). The amplified products coding for VP7Δ, VP4Δ, and fljB were cloned into pBluescript KS+ (pBS; Stratagene) and confirmed by DNA sequencing (McGill University and Génome Québec Innovation Centre, Montreal, Canada).

Table 1 Oligonucleotide primers used in this study

Primers used for the generation of constructs in pTrcHisB.	
VP7_WT(F)	CGCAGATCTATGTATCCAACTGAAGCAAG
VP7_WT(R)	GCGCAAGCTTTCACTACTCGAGTACTCTATAATAAAAAGCTG
VP4_WT(F)	GCGGAGATCTGCCACCATGGCTTCACTCATTTATAG
VP4_WT(R)	GGTATTAGATCTTCATCACCCTCCATTATAGCTAAAATTGTTCACTCCA
fljB_WT(F)	GAGAGGTACCATGGCACAAGTAATCAACACTAAC
fljB_WT(R)	GAGAGAATTCCTAACGTAACAGAGACAGCACGTT
Primers used for the generation of single and dimeric constructs in pRTL2. Blunt PCR fragments were cloned into pBluescript (SK) before cloning in the pRTL2 vector.	
Single constructs	
GFP(F)	GAGACCATGGGTAAAGGAGAACT
GFP(R)	GAGATCTAGAGGGGAGCTCCTAGATAGATCTGTATAGTTCAT
VP7(F)	GAGACCATGGGGTACGGTATTGAGTACACCACC
VP7(R)	GAGATCTAGAGGGGAGCTCCTAAAGCTCATCCTTTTCACTAACACGGTAG
VP4(F)	GAGACCATGGCTTCTCTTATCTACCGTCAG
VP4(R)	GAGATCTAGAGGGGAGCTCCTAAAGCTCATCCTTTTCACTACCACCGTTG
fljB(F)	GAGATCTAGAATGGCACAAGTAATCAACACTAAC
fljB(R)	GAGAGAATTCGGGAGCTCCTAAAGCTCATCCTTTTCACTACGTAACAGAGACAGCACGTT
Dimeric constructs	
VP7_1(F)	GAGATCTAGAATGTACGGTATTGAGTACACCACC
VP7_1(R)	GAGAGAATTCGGGAGATCTAACACGGTAGTAGAAAGCAGC
VP7_2(F)	GAGAAGATCTGCCACCATGTACGGTATTGAGTACACCACC
VP7_2(R)	GAGAGAATTCGGGGAGCTCCTAAAGCTCATCCTTTTCACTAACACGGTAGTAGAAAGCAGC
VP4_1(F)	GAGATCTAGAATGGCTTCTCTTATCTACCGTCAG
VP4_1(R)	GAGAGAATTCGGGGGATCCACCACCGTTGTAAGAGAA
VP4_2(F)	GAGAGGATCCGGGGCCACCATGGCTTCTCTTATCTACCGTCAG
VP4_2(R)	GAGAGAATTCGGGGAGCTCCTAAAGCTCATCCTTTTCACTACCACCGTTGTAAGAGAA
fljB_2(F)	GAGAGGATCCGCCACCATGGCACAAGTAATCAACACTAAC
fljB_2(R)	GAGAGAATTCGGGAGCTCCTAAAGCTCATCCTTTTCACTACGTAACAGAGACAGCACGTT
Primers used for RT-PCR analyses.	
RT-VP7(F)	GTGGTGGCAAGTTTTCTACACCATCG
RT-VP8(F)	GGCTGCTAACTACCAGTACAACTACCTTAGGG
RT-fljB(F)	CCGAAGTTTCCAACATGTCTCGCG
RT-CaMVterm(R)	GCATGCCTGCAGGTCACTGGATT
RT-Actin2(F)	TCAGATGCCCAGAAGTGTGTT
RT-Actin2(R)	CCGTACAGATCCTTCCTGATAT

The restriction enzyme cutting sites used for cloning are underlined

Production of Rabbit His::VP7Δ, His::VP4Δ, and His::fljB Antisera

To produce recombinant proteins for antibody generation in rabbit, the VP7Δ, VP4Δ, and fljB cDNAs were cloned into the pTrcHisB expression vector (Invitrogen) to generate N-terminal $(His)_6$-tagged fusions. These constructs were transformed into DH5α *E. coli* cells by heat shock, and recombinant protein expression was induced with 1 mM IPTG for 5 h. Proteins were purified on Ni-NTA-His-Bind® resin (Novagen) under denaturing conditions according to the manufacturer's manual. The purified His::VP7Δ, His::VP4Δ, and His::fljB proteins were dialyzed against phosphate-buffered saline (PBS) (pH 7.3), then quality was assessed by SDS-PAGE and immunoblotting using an HRP-coupled goat anti-His antibody (1:20,000) (QIAGEN) with the Western Lightning® chemiluminescence reagent (Perkin-Elmer). The identity of the proteins was confirmed by MALDI time-of-flight tandem mass spectrometry (McGill University and Génome Québec Innovation Centre), using the MASCOT software (Matrix Science). Protein concentrations were determined with the Protein Assay® Kit (Bio-Rad). Polyclonal antibodies were raised by immunizing New Zealand white

rabbits with the purified proteins according to standard procedures.

Generation of Constructs for Expression in Plants

To ensure optimal expression in plants, the coding sequences of the full-length VP7 (GenBank accession no. AAA47342) and VP4 (acc. no. AAA66953) genes from the human rotavirus Wa strain were optimized for codon usage in dicotyledonous plants and chemically synthesized (GeneArt, Regensburg Germany; Fig. 1). We also modified sequences that may destabilize mRNA, and mutated endonuclease restriction sites *Xba*I, *Sac*I, and *Eco*RI when present, to facilitate cloning.

Constructs VP7, VP4Δ, fljB, VP7::VP4Δ, VP4Δ::VP7, VP7::fljB, and VP4Δ::fljB (Fig. 2a) were generated using the optimized full-length VP7 and truncated VP4Δ sequences, and the native fljB sequence. Fragments were amplified by PCR using specific primers (Table 1) and cloned individually in pBS. The individual fragments generated by restriction digestion were ligated to form the various dimeric combinations. The flexible hinge peptide GSAT was included between the various moieties to facilitate proper protein folding and maximize antigenicity. The single and dimeric sequences were then cloned into the pRTL2 vector [20], in an expression cassette consisting of the cauliflower mosaic virus (CaMV) double 35S promoter, the tobacco etch virus untranslated leader sequence at the 5′ end, and the SEKDEL in-frame sequence (endoplasmic reticulum retention signal) and CaMV 35S transcription terminator at the 3′ end. All constructs were confirmed by DNA sequencing. The plant expression vectors were generated by the insertion of each expression cassette in the *Pst*I site of pCambia1380.

Agroinfiltration of *Nicotiana benthamiana*

Nicotiana benthamiana plants were grown in growth chambers in a 1:1:1 mixture of black earth, ProMix (Premier) and vermiculite, at a temperature regime of 25°C day/21°C night with a 16-h photoperiod. After 2 weeks, plantlets were potted individually and grown under the same conditions for two additional weeks.

For transient expression assays, the pCambia constructs were first electroporated in *Agrobacterium tumefaciens* strain AGL1. *Agrobacteria* were grown in LB medium containing ampicillin and tetracyclin until they reached an OD_{600} of 0.8. *Agrobacteria* suspensions were centrifuged and resuspended in the MMA infiltration medium [21] containing 20 μM acetosyringone. Each suspension was mixed 1:1 with a suspension of *Agrobacteria* carrying the P19 suppressor of post-transcriptional gene silencing to enhance transient expression of the heterologous constructs. Cultures were incubated 2 h at room temperature, then infiltrations were done on the lower side of leaves. Plants were covered with a protective hood and returned to the growth chamber for 24 h. Hoods were removed and plants were grown up to 10 days before being harvested. Plants at various developmental stages at the time of infiltration and various post-infection conditions were tested to determine the optimal conditions for maximal protein accumulation.

Aerial parts of the plants were harvested, frozen in liquid nitrogen and ground into a fine powder. Proteins were extracted and precipitated with the trichloroacetic acid (TCA)–acetone method [22] with some modifications. The powder was homogenized in cold acetone containing 10% TCA 0.07% 2-mercapto-ethanol. After precipitation at −20°C, the pellet was recovered by centrifugation and washed at least three times with cold acetone until the supernatant was colorless. The final pellet was dried to remove traces of acetone and proteins were solubilized in PBS containing 8 M urea. Slurries were centrifuged at 17,000×*g* for 20 min and the supernatant was dialyzed against a series of PBS solutions containing decreasing urea concentrations. Proteins were concentrated 20-fold by ethanol precipitation (80% v/v final concentration), solubilized in water and stored at −80°C.

Molecular Analyses

For immunoblot analyses, proteins were separated on 12% SDS-polyacrylamide gels and transferred onto PVDF membranes. Blots were blocked with PBS–Tween 20 (0.05% v/v) containing 5% (w/v) powdered skimmed milk, then incubated with the rabbit anti-His::VP7Δ (1:5000), anti-His::VP4Δ (1:5000) or anti-His::fljB (1:20,000) antibodies. Detection was performed with an HRP-coupled goat anti-rabbit-IgG antibody (1:15,000; Santa Cruz Biotechnology) and the Western Lightning® chemiluminescence reagent (Perkin-Elmer). To determine the levels of proteins expressed in the infiltrated plants, immunoblots of different dilutions of plant samples and known amounts of recombinant His-tagged proteins expressed in *E. coli* were analyzed by densitometry of the X-ray films with the Quantity One software (Bio-Rad).

For RT-PCR analyses, total RNA was isolated from agro-infiltrated *Nicotiana benthamiana* leaves using the TRIzol Reagent (Invitrogen), then reverse-transcribed using SuperScript II Reverse Transcriptase (Invitrogen). The transgene transcripts were amplified by PCR (about 250 bp each) using specific primers (Table 1). PCR amplification of actin2 transcripts was used as a control. PCR products were analyzed by electrophoresis on 0.8% agarose/ethidium bromide gels.

Fig. 1 Alignments of optimized and native rotaviral gene sequences. **a** The VP4 native sequence is from acc. no. AAA66953. **b** The VP7 native sequence is from acc. no. AAA47342. The sequences were optimized to match the codon usage in dicotyledonous plants, to modify sequences that may destabilize mRNA, and to mutate endonuclease restriction sites *Xba*I, *Sac*I, and *Eco*RI (to facilitate cloning)

A

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optimized_VP4     ATGGCTTCTCTTATCTACCGTCAGTTGCTTACCAACTCTTACTCTGTTGATCTTCACGAT 60
native_VP4        ATGGCTTCACTCATTTATAGACAGCTTCTCACTAATTCATATTCAGTAGATTTACATGAT 60
                  ******** ** ** **  * *** * ** ** ** ** ** ** ** *** * ** ***

optimized_VP4     GAGATCGAGCAGATTGGTTCTGAAAAGACCCAGAACGTTACCATTAACCCTTCTCCATTC 120
native_VP4        GAAATAGAGCAAATTGGATCAGAAAAAACTCAAAATGTAACTATAAATCCGAGTCCATTT 120
                  ** ** ***** ***** ** ***** ** ** ** ** ** ** ** **   ******

optimized_VP4     GCTCAAACTCGTTACGCTCCTGTTAATTGGGGTCACGGAGAGATTAACGATTCTACCACC 180
native_VP4        GCACAGACTAGATATGCTCCAGTCAATTGGGGTCATGGAGAGATAAATGATTCGACTACA 180
                  ** ** *** * ** ***** ** *********** ******** ** ***** ** **

optimized_VP4     GTTGAGCCTATGCTTGATGGTCCTTACCAACCTACTACTTTCACCCCTCCTAACGATTAC 240
native_VP4        GTAGAACCAATGTTAGATGGTCCTTATCAGCCAACTACATTTACTCCACCTAATGATTAT 240
                  ** ** ** *** * *********** ** ** ***** ** ** ** ***** *****

optimized_VP4     TGGATACTTATCAACTCTAACACCAACGGTGTTGTTTACGAGTCTACCAACAACTCTGAT 300
native_VP4        TGGATACTTATTAATTCAAATACAAATGGAGTAGTATATGAAAGTACAAATAATAGTGAC 300
                  *********** ** ** ** ** ** ** ** ** ** **   *** ** **   ***

optimized_VP4     TTCTGGACCGCTGTTGTTGCTATTGAGCCTCACGTTAACCCTGTTGATAGGCAGTACACC 360
native_VP4        TTTTGGACTGCAGTCGTTGCTATTGAACCGCACGTTAACCCAGTAGATAGACAATATACG 360
                  ** ***** ** ** *********** ** *********** ** ***** ** ** **

optimized_VP4     ATCTTCGGTGAGTCTAAGCAGTTCAACGTTTCTAACGATTCTAACAAGTGGAAGTTCCTT 420
native_VP4        ATATTTGGTGAAAGTAAGCAATTTAATGTGAGTAACGATTCAAATAAATGGAAGTTTTTA 420
                  ** ** *****   ****** ** ** **   ********* ** ** ********   *

optimized_VP4     GAGATGTTCAGGTCATCTTCTCAGAACGAGTTCTACAACAGGCGTACCCTTACTTCTGAT 480
native_VP4        GAAATGTTTAGAAGCAGTAGTCAAAATGAATTTTATAATAGACGTACATTAACTTCTGAT 480
                  ** ***** **      *  *** ** ** ** ** ** ** *****  * *********

optimized_VP4     ACCCGTTTCGTTGGTATCCTTAAGTACGGTGGTCGTGTTTGGACTTTCCACGGTGAAACT 540
native_VP4        ACTAGATTTGTAGGAATATTGAAATATGGTGGAAGAGTATGGACATTTCATGGTGAAACA 540
                  **  * ** ** ** **  * ** ** *****  * ** ***** ** ** ********

optimized_VP4     CCTAGAGCTACCACCGATTCTTCATCTACCGCTAACCTTAACAACATCTCTATCACCATC 600
native_VP4        CCAAGAGCTACTACTGACAGTTCAAGTACTGCAAATTTAAATAATATATCAATTACAATT 600
                  ** ******** ** **   ****  *** ** **  * ** ** ** ** ** ** **

optimized_VP4     CACTCTGAGTTCTACATCATCCCTAGGTCTCAAGAGTCTAAGTGCAACGAGTACATCAAC 660
native_VP4        CATTCAGAATTTTACATTATTCCAAGGTCCCAAGAATCTAAATGTAATGAATATATTAAT 660
                  ** ** ** ** ***** ** ** ***** ***** ***** ** ** ** ** ** **

optimized_VP4     AACGGACTTCCTCCTATTCAAAACACCAGGAACGTTGTTCCTCTTCCTCTTTCTTCTCGT 720
native_VP4        AATGGTCTGCCACCAATTCAAAATACTAGAAATGTAGTTCCATTGCCATTATCATCTAGA 720
                  ** ** ** ** ** ******** ** ** ** ** *****  * **  * ** *** *

optimized_VP4     TCTATCCAGTACAAGAGGGCTCAAGTTAACGAGGATATTATCGTTTCTAAGACCTCACTT 780
native_VP4        TCGATACAGTATAAGAGAGCACAAGTTAATGAAGACATTATAGTTTCAAAAACTTCATTA 780
                  ** ** ***** ***** ** ******** ** ** ***** ***** ** ** *** *

optimized_VP4     TGGAAAGAGATGCAGTACAACAGGGATATTATCATCAGGTTCAAGTTCGGTAACTCTATC 840
native_VP4        TGGAAAGAAATGCAGTATAATAGGGATATTATAATTAGATTTAAATTTGGTAATAGTATT 840
                  ******** ******** ** *********** ** ** ** ** ** *****   ***

optimized_VP4     GTTAAGATGGGTGGTCTTGGTTACAAGTGGAGTGAGATCTCTTACAAGGCTGCTAACTAC 900
native_VP4        GTAAAAATGGGAGGACTAGGTTATAAATGGTCTGAAATATCATATAAGGCAGCAAATTAT 900
                  ** ** ***** ** ** ***** ** ***  *** ** ** ** ***** ** ** **

optimized_VP4     CAGTACAACTACCTTAGGGATGGTGAACAAGTTACTGCTCACACCACCTGTTCTGTTAAC 960
native_VP4        CAATATAATTACTTACGTGACGGTGAACAAGTAACCGCACACACCACTTGTTCAGTAAAT 960
                  ** ** ** *** *  * ** *********** ** ** ******** ***** ** **

optimized_VP4     GGTGTTAACAACTTCTCTTACAACGGTGGTTTCCTTCCTACCGATTTCGGTATCTCTCGT 1020
native_VP4        GGAGTGAACAATTTTAGCTATAATGGAGGGTTTCTACCCACTGATTTTGGTATTTCAAGG 1020
                  ** ** ***** **    ** ** ** ** ** ** ** ** ***** ***** **  *

optimized_VP4     TACGAGGTTATCAAAGAGAACTCTTACGTTTACGTTGATTACTGGGATGATTCTAAGGCT 1080
native_VP4        TATGAAGTTATTAAAGAGAATTCCTATGTATATGTAGACTATTGGGATGATTCAAAAGCA 1080
                  ** ** ***** ******** ** ** ** ** ** ** ** *********** ** **

optimized_VP4     TTCAGGAACATGGTTTACGTTAGATCTCTTGCTGCTAACTTGAACTCTGTTAAGTGCACC 1140
native_VP4        TTTAGAAATATGGTATATGTTAGATCATTAGCAGCTAATTTGAATTCAGTGAAATGTACA 1140
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Mice Immunization

Six-week-old female BALB/c mice were obtained from Charles River Canada. Groups of eight mice were immunized subcutaneously with 50 µl of each concentrated extract emulsified with an equal volume of Stimune™ adjuvant (Cedi Diagnostics). Since the expression level and solubility varied between the target proteins, the maximum quantity of protein administered per dose varied between extracts: VP7 (0.425 µg), VP4Δ (2.5 µg), fljB (2.025 µg),

Fig. 1 continued

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optimized_VP4      GGTGGTTCTTACAACTTCTCTATCCCTGTTGGTGCTTGGCCTGTTATGAATGGTGGTGCT 1200
native_VP4         GGTGGAAGTTATAATTTTAGTATACCAGTAGGTGCATGGCCAGTAATGAATGGTGGCGCT 1200
                   *****   *** ** **   *** ** ** ***** ***** ** *********** ***

optimized_VP4      GTTTCTCTTCACTTCGCTGGTGTTACCCTTTCTACCCAGTTCACCGATTTCGTTTCTCTT 1260
native_VP4         GTTTCGTTGCATTTTGCCGGAGTTACATTATCCACGCAATTTACTGATTTTGTATCATTG 1260
                   *****  * ** ** ** ** *****  * ** ** ** ** ** ***** ** **  *

optimized_VP4      AACTCTCTTAGGTTCAGGTTCTCTCTTACCGTTGATGAGCCTCCTTTCTCTATTCTTAGG 1320
native_VP4         AATTCACTACGATTTAGATTTAGTTTGACAGTTGATGAACCACCTTTCTCAATATTGAGA 1320
                   ** ** **  * ** ** **   * * ** ******** ** ******** **  * **

optimized_VP4      ACCAGGACCGTTAACCTTTATGGACTTCCTGCTGCTAATCCTAACAACGGTAACGAGTAC 1380
native_VP4         ACACGTACAGTGAATTTGTACGGATTACCAGCCGCTAATCCAAATAATGGAAATGAATAC 1380
                   **  * ** ** **  * ** *** * ** ** ******** ** ** ** ** ** ***

optimized_VP4      TACGAGATCTCTGGTAGGTTCTCACTTATCTACCTTGTTCCTACCAACGATGATTACCAG 1440
native_VP4         TACGAAATATCAGGAAGGTTTTCACTCATTTATTTAGTTCCAACTAATGATGATTATCAG 1440
                   ***** ** ** ** ***** ***** ** **  * ***** ** ** ******** ***

optimized_VP4      ACCCCTATCATGAACTCAGTTACTGTTAGGCAGGATCTTGAGAGGCAACTTACCGATCTT 1500
native_VP4         ACTCCAATTATGAATTCAGTGACAGTAAGACAAGATTTAGAGCGCCAACTTACTGATTTG 1500
                   ** ** ** ***** ***** ** ** ** ** *** * *** * ******** *** *

optimized_VP4      AGGGAAGAGTTCAACTCTCTTTCTCAAGAGATCGCTATGGCTCAGTTGATCGATCTTGCT 1560
native_VP4         CGAGAAGAATTTAACTCATTGTCACAAGAAATAGCTATGGCACAATTGATTGATTTAGCA 1560
                    * ***** ** *****  * ** ***** ** ******** ** ***** *** * **

optimized_VP4      CTTCTTCCTCTTGATATGTTCTCAATGTTCTCTGGTATCAAGTCTACCATCGATCTTACC 1620
native_VP4         CTGTTGCCTCTAGATATGTTTTCCATGTTTTCAGGAATTAAAGTACAATTGATTTAACT 1620
                   **  * ***** ******** ** ***** ** ** ** **   *** ** *** * **

optimized_VP4      AAGTCTATGGCTACCTCTGTTATGAAGAAGTTCAGGAAGTCTAAGCTTGCTACCTCTATC 1680
native_VP4         AAATCAATGGCGACTAGTGTAATGAAGAAATTTAGAAAATCAAAATTAGCTACATCAATT 1680
                   ** ** ***** **   *** ******** ** ** ** ** **  * ***** ** **

optimized_VP4      TCTGAGATGACCAACTCACTTTCTGATGCTGCTTCATCTGCTTCTAGGAACGTTTCTATC 1740
native_VP4         TCAGAAATGACTAATTCATTGTCAGATGCTGCTTCATCAGCATCAAGAAACGTTTCTATT 1740
                   ** ** ***** ** *** * ** ************** ** ** ** ***********

optimized_VP4      CGTTCTAACCTTTCTGCTATCTCTAACTGGACCAATGTTTCAAACGATGTTTCTAACGTT 1800
native_VP4         AGATCGAATTTATCTGCGATCTCAAATTGGACTAATGTTTCAAATGATGTGTCAAACGTA 1800
                    * ** **  * ***** ***** ** ***** *********** ***** ** *****

optimized_VP4      ACCAACTCTCTTAACGATATTTCTACCCAGACCTCTACCATCTCTAAGAAGTTCCGTCTT 1860
native_VP4         ACTAATTCATTGAACGATATTTCAACACAAACGTCTACAATTAGTAAGAAATTTAGATTA 1860
                   ** ** **  * *********** ** ** ** ***** **   ****** **  *  *

optimized_VP4      AAAGAGATGATCACCCAGACTGAGGGAATGTCTTTCGATGATATTTCTGCTGCTGTTCTT 1920
native_VP4         AAAGAAATGATTACTCAAACTGAAGGAATGAGCTTTGACGACATTTCAGCAGCTGTACTA 1920
                   ***** ***** ** ** ***** ******   ** ** ** ***** ** ***** **

optimized_VP4      AAGACCAAGATCGATATGTCAACCCAGATCGGAAAGAACACCCTTCCTGATATTGTTACT 1980
native_VP4         AAAACAAAAATAGATATGTCTACTCAAATTGGAAAAAATACTTTACCTGACATAGTTACA 1980
                   ** ** ** ** ******** ** ** ** ***** ** **  * ***** ** *****

optimized_VP4      GAGGCTTCTGAGAAGTTCATCCCTAAGAGGTCTTACCGTATCTTGAAGGATGATGAGGTT 2040
native_VP4         GAGGCATCTGAGAAATTTATTCCAAAACGATCATATCGAATATTAAAGGATGATGAAGTA 2040
                   ***** ******** ** ** ** **  * ** ** ** ** ** *********** **

optimized_VP4      ATGGAGATCAACACCGAGGGAAAGTTCTTCGCTTACAAGATCAACACCTTCGATGAGGTT 2100
native_VP4         ATGGAAATTAATACTGAAGGAAAATTCTTTGCATACAAAATTAATACATTTGATGAAGTG 2100
                   ***** ** ** ** ** ***** ***** ** ***** ** ** ** ** ***** **

optimized_VP4      CCATTCGATGTTAACAAGTTCGCTGAGTTGGTTACCGATTCTCCTGTTATCTCTGCTATC 2160
native_VP4         CCATTCGATGTAAATAAATTCGCTGAACTAGTAACAGATTCTCCAGTTATATCAGCGATA 2160
                   *********** ** ** ********  * ** ** ******** ***** ** ** **

optimized_VP4      ATTGATTTCAAGACCCTTAAGAACCTTAACGATAACTACGGTATCACTAGGACTGAGGCT 2220
native_VP4         ATCGATTTTAAGACATTGAAAAATTTAAATGATAATTATGGAATCACTCGTACAGAAGCG 2220
                   ** ***** *****  * ** **  * ** ***** ** ** ****** * ** ** **

optimized_VP4      CTTAACCTTATCAAGTCAAACCCTAACATGCTTAGGAACTTCATCAACCAGAACAACCCT 2280
native_VP4         TTAAATTTAATTAAATCGAATCCAAATATGTTGCGTAATTTCATTAATCAAAATAATCCA 2280
                    * **  * ** ** ** ** ** ** *** *  * ** ***** ** ** ** ** **

optimized_VP4      ATCATCAGGAACAGGATCGAGCAACTTATCCCCGGGTCTGAGAAGGATGAGCTTTAGTAA 2340
native_VP4         ATTATAAGGAATAGAATTGAACAGTTAATACTACAATGTAAATTG--TGAG--------- 2329
                   ** ** ***** ** ** ** **  * ** *     * * *   *  ****
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VP7::fljB (15.985 μg), and VP4Δ::fljB (6.125 μg). This approach was used instead of adjusting all target proteins to the lowest level of expression, thus improving the likelihood of generating an immune response. An extract of plant-expressed green fluorescent protein (GFP) was used as a negative control. Booster immunizations were

Fig. 1 continued

B

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optimized_VP7      ATGTACGGTATTGAGTACACCACCATCCTTATCTTCCTTATCAGTATCATCCTTCTTAAC 60
native_VP7         ATGTATGGTATTGAATATACCACAATTCTAATCTTTTTGATATCAATCATTCTACTCAAC 60
                   ***** ******** ** ***** ** ** *****  * **    ***** ** ** ***

optimized_VP7      TACATCCTTAAGTCTGTTACCAGGATCATGGATTACATCATCTACCGTTTCCTTTTGATC 120
native_VP7         TATATATTAAAATCAGTGACTCGAATAATGGACTACATTATATATAGATTTTTGTTGATT 120
                   ** **  * ** ** ** **  * ** ***** ***** ** **  * **  * *****

optimized_VP7      ACCGTTGCTTTGTTCGCTCTTACCAGGGCTCAAAACTACGGTCTTAACCTTCCTATCACC 180
native_VP7         ACTGTAGCATTATTTGCTTTGACAAGAGCTCAGAATTATGGACTTAACTTACCAATAACA 180
                   ** ** ** ** ** *** * ** ** ***** ** ** ** ****** * ** ** **

optimized_VP7      GGTTCTATGGATGCTGTTTACACCAACTCTACCCAGGAAGAGGTTTTCCTTACCTCTACC 240
native_VP7         GGATCAATGGACGCTGTATATACTAACTCTACTCAAGAAGAAGTGTTTCTAACTTCTACG 240
                   ** ** ***** ***** ** ** ******** ** ***** ** ** ** ** *****

optimized_VP7      CTTTGCCTTTACTACCCTACTGAGGCTTCTACCCAGATCAATGATGGTGATTGGAAGGAT 300
native_VP7         TTATGTCTGTATTATCCAACTGAAGCAAGTACTCAAATCAATGATGGTGACTGGAAAGAC 300
                    * ** ** ** ** ** ***** **   *** ** ************** ***** **

optimized_VP7      TCTCTTTCTCAGATGTTCCTTACCAAGGGATGGCCTACTGGTTCTGTTTACTTCAAAGAG 360
native_VP7         TCATTGTCGCAAATGTTTCTTACAAAGGGTTGGCCAACAGGATCTGTTTACTTTAAAGAG 360
                   **  * ** ** ***** ***** ***** ***** ** ** *********** ******

optimized_VP7      TACTCTAACATCGTTGATTTCTCTGTTGATCCTCAGCTTTACTGCGATTACAACCTTGTT 420
native_VP7         TACTCAAATATTGTTGATTTTTCTGTTGACCCACAGCTGTATTGTGACTATAATTTAGTA 420
                   ***** ** ** ******** ******** ** ***** ** ** ** ** **  * **

optimized_VP7      CTTATGAAGTACGATCAGTCTCTTAAGCTTGATATGTCTGAGCTTGCTGATCTTATCCTT 480
native_VP7         CTTATGAAATATGACCAAAGTCTTAAATTAGATATGTCAGAGTTAGCTGATTTAATATTG 480
                   ******** ** ** **   ******  * ******** *** * ****** * **  *

optimized_VP7      AACGAGTGGTTGTGCAACCCTATGGATGTTACCCTTTACTACTACCAGCAGTCTGGTGAA 540
native_VP7         AATGAATGGTTATGTAACCCAATGGATGTAACATTATACTATTATCAACAATCGGGAGAA 540
                   ** ** ***** ** ***** ******** **  * ***** ** ** ** ** ** ***

optimized_VP7      TCTAACAAGTGGATCTCTATGGGATCTTCTTGCACCGTTAAAGTTTGCCCTCTTAACACT 600
native_VP7         TCAAATAAGTGGATATCGATGGGATCATCATGTACCGTGAAAGTGTGTCCGCTAAATACA 600
                   ** ** ******** ** ******** ** ** ***** ***** ** ** ** ** **

optimized_VP7      CAGACCCTTGGTATTGGTTGTCAGACCACCAACGTTGATTCTTTCGAGATGATCGCTGAG 660
native_VP7         CAAACGTTAGGGATAGGTTGTCAAACAACAAACGTAGACTCATTTGAAATGATTGCTGAG 660
                   ** **  * ** ** ******** ** ** ***** ** ** ** ** ***** ******

optimized_VP7      AACGAGAAGCTTGCTATCGTTGATGTTGTTGATGGTATCAACCACAAGATCAACCTTACT 720
native_VP7         AATGAGAAATTAGCTATAGTGGATGTCGTTGATGGGATAAATCATAAAATAAATTTAACA 720
                   ** *****  * ***** ** ***** ******** ** ** ** ** ** **  * **

optimized_VP7      ACCACCACTTGCACCATTAGGAACTGCAAGAAGCTTGGTCCTAGGGAAAACGTTGCTGTT 780
native_VP7         ACTACGACATGTACTATTCGAAATTGTAAGAAATTAGGTCCAAGAGAAAATGTAGCTGTA 780
                   ** ** ** ** *** * ** ** *****  * ***** ** ***** ** *****

optimized_VP7      ATCCAAGTTGGTGGTTCTAACGTTCTTGATATTACCGCTGATCCTACTACTAACCCTCAA 840
native_VP7         ATACAAGTTGGTGGTTCTAATGTGTTAGACATAACAGCAGATCCAACAACTAATCCACAA 840
                   ** ***************** **  * ** ** ** ** ***** ** ***** ** ***

optimized_VP7      ACCGAGAGGATGATGAGGGTTAACTGGAAGAAGTGGTGGCAAGTTTTCTACACCATCGTT 900
native_VP7         ACTGAGAGAATGATGAGAGTGAATTGGAAAAAGTGGTGGCAAGTATTTTATACTATAGTA 900
                   ** ***** ******** ** ** ***** ************** ** ** ** ** **

optimized_VP7      GATTACATCAACCAGATCGTTCAGGTTATGTCTAAGAGGTCTCGTTCTCTTAACTCTGCT 960
native_VP7         GATTATATTAATCAAATTGTACAGGTAATGTCCAAAAGATCAAGATCATTAAATTCTGCA 960
                   ***** ** ** ** ** ** ***** ***** ** ** **  * **  * ** *****

optimized_VP7      GCTTTCTACTACCGTGTTCCCGGGAGTGAAAAGGATGAGCTTTAGTAG 1008
native_VP7         GCTTTTTATTATAGAGTATAG--------------------------- 981
                   ***** ** **  * **
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administered 17 and 34 days following primary immunizations. Blood samples were collected at 48 days, then sera were stored at −80°C.

Assessment of Antigen-Specific Antibody and Cellular Responses

Humoral response in mice was assayed by immunoblot, indirect immunofluorescence and indirect ELISA analyses of sera. For immunoblot analysis, 0.25–2 μg of each His-tagged recombinant protein (VP7Δ, VP4Δ or fljB) was separated on 12% gels, transferred onto PVDF membranes and probed with each pooled mouse serum (1:1000). Signals were detected as described in "Molecular Analyses" except that an HRP-coupled goat anti-mouse IgG-Fc antibody (1:10,000) (Bethyl Laboratories) was used.

For indirect ELISA (enzyme linked immunosorbent assay) analysis, high binding Costar 96-well plates

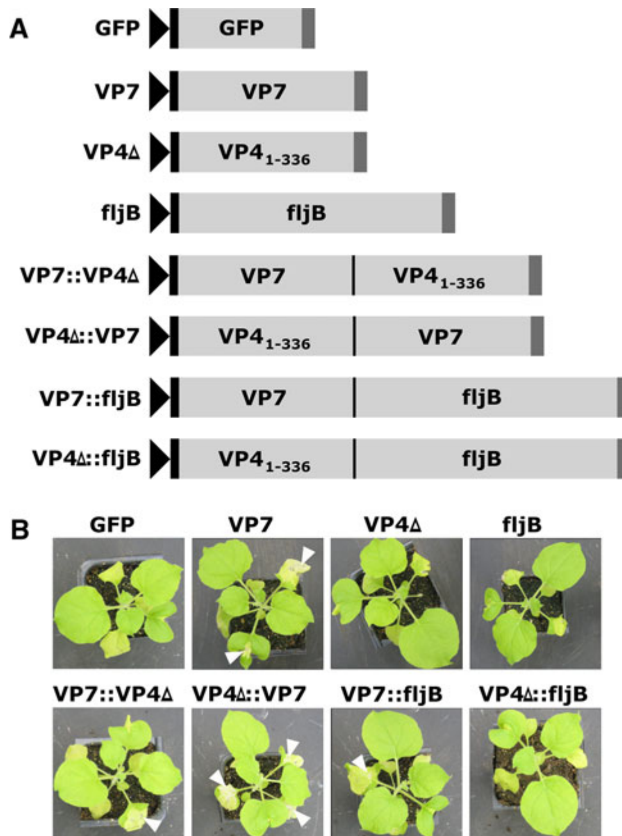

Fig. 2 Transient expression of rotaviral VP and bacterial flagellin proteins in *Nicotiana benthamiana*. **a** Constructs used for expression of HRV and *Salmonella* proteins in *Nicotiana benthamiana* leaves. The sequence encoding the SEKDEL endoplasmic reticulum retention signal was inserted at the 3′ end of the coding sequence. *Black arrowheads*, double Cauliflower Mosaic Virus (CaMV) 35S promoter. *Black boxes*, Tobacco Etch Virus 5′ untranslated region. *Gray boxes*, coding regions. *Dark gray boxes*, CaMV 35S terminator. **b** *Nicotiana benthamiana* plants following agro-infiltration. Three week-old plants were agro-infiltrated at day 0 with *Agrobacterium tumefaciens* strain AGL1 carrying the various constructs in the pCambia1380 vector, and pictures were taken at various times post-infiltration. Only the pictures at day 10 are shown. A GFP construct was used as a negative control. *Arrowheads* indicate damaged leaves

(Corning) were incubated overnight with 1 μg of His-tagged recombinant protein (VP7Δ, VP4Δ or fljB) per well. Plates were then blocked with PBS Tween 0.2%, BSA 0.5%, incubated with each pooled mouse sera for 2 h, washed with PBS Tween 0.2%, and incubated with secondary HRP-coupled goat anti-mouse IgG-Fc antibody (1:10,000). Detection was achieved with 3,3′,5,5′-tetramethylbenzidine (TMB) dihydrochloride according to the manufacturer's procedure (Sigma). One hour after adding the reagents, the reactions were stopped with 0.2 M sulphuric acid and plates were read at 655 nm. A signal was considered positive when the mean value of the duplicates was twofold higher than the GFP control group. This experiment was repeated twice.

Results and Discussion

Production of Recombinant Proteins in *E. coli*

The recombinant His-tagged VP7 coding sequence was found to be toxic to *E. coli* (data not shown), as previously reported [23]. Alternatively, the truncated VP7Δ fragment was selected on the basis of antigenicity index and surface probability analyses. This fragment bears the three major antigenic sites containing dominant neutralization epitopes, designated A (amino acids 87–99), B (amino acids 145–150), and C (amino acids 211–223) [23]. The truncated His::VP7Δ protein was successfully expressed as a 31 kDa recombinant protein (Fig. 3a) at a yield of 4 mg/l of bacterial culture.

A truncated fragment of the 776 amino acids VP4 protein was generated to reduce the length of transcript and recombinant protein and thus facilitate expression. The

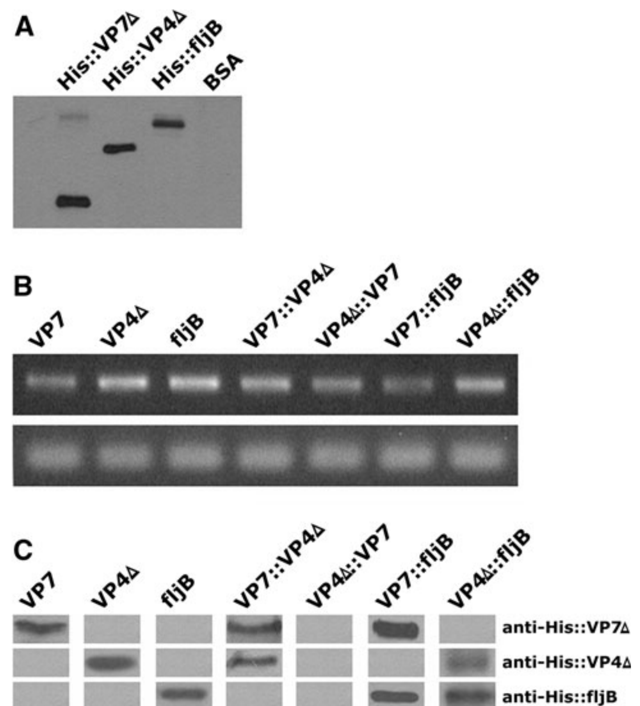

Fig. 3 Expression in *Escherichia coli* and *Nicotiana benthamiana*. **a** Recombinant His-tagged proteins expressed in *Escherichia coli* were analyzed by immunoblotting using an HRP-coupled anti-His antibody and signals were detected by chemiluminescence. BSA, bovine serum albumin used as a negative control. **b** Target transcripts expressed in agro-infiltrated *Nicotiana benthamiana* leaves were reverse-transcribed using SuperScript II reverse transcriptase, then amplified by PCR using specific primers to generate fragments of about 250 bp. *Lower panel*, Actin 2 used as a loading control. No signal was detected in leaves agro-infiltrated with the negative control constructs GFP or with the empty vector (data not shown). **c** Target proteins were detected by immunoblot analysis using anti-His::VP7Δ, anti-His::VP4Δ or anti-His::fljB antibodies. Exposure times varied between each construct and antibody

selected VP4Δ region contains the VP8* subunit that presents linear and conformational neutralizing epitopes [11], the cleavage site between the VP8* and VP5* subunits, and the amino-terminal region of VP5* containing the DGE sequence motif essential for virus attachment to target cells [24]. The 41 kDa His::VP4Δ protein was produced as a homogeneous, soluble protein at a yield of 2 mg/l of bacterial culture (Fig. 3a).

Sequence alignment of various fljB genes from distinct types and strains of *Salmonella* identified two conserved domains at the NH_2 and COOH termini [25] known to trigger PAMP recognition pathways [26]. We therefore cloned a DNA fragment encoding a 300 amino acids fljB protein containing these conserved domains from *Salmonella typhimurium*. A 53 kDa His-tagged fljB protein was expressed in *E. coli* with a yield similar to the His::VP7Δ and His::VP4Δ proteins (Fig. 3a).

The recombinant His-tagged VP7Δ, VP4Δ, and fljB proteins were used to raise antibodies in rabbits. Sera of immunized rabbits contained high titers of specific antibodies that recognize recombinant proteins produced in both *E. coli* (data not shown) and transformed plants (Fig. 3c). This demonstrates that the protein fragments selected are able to generate an immune response in animals.

Transient Expression in *Nicotiana benthamiana*

To rapidly produce proteins in plants, the single antigen and multiple antigen combinations were transiently expressed in *Nicotiana benthamiana*. The agro-infiltration technique allows protein expression in leaves infected with *Agrobacterium tumefaciens* carrying the various constructs. Although research groups often follow with the generation of stable transformants, it is now acknowledged that the transient system can be used for large scale production of recombinant proteins in plants [27].

Coding sequences of the VP7 and VP4 genes were optimized for codon usage and expression in dicotyledonous plants. The synthetic genes encode protein sequences identical to those encoded by the native VP7 and VP4 genes (Fig. 1). The individual synthetic full-length VP7, truncated VP4Δ, and native fljB sequences were assembled into various combinations, namely VP7::VP4Δ, VP4Δ::VP7, VP7::fljB, and VP4Δ::fljB (Fig. 2a). Each single and dimeric coding region was fused to the 5′ untranslated region of the tobacco etch virus, which stabilizes mRNAs on ribosomes and accentuates transcription. Transformed leaves that accumulate the antigens showed no visible damage, except for constructs VP7 and VP4Δ::VP7, where mild necrosis was observed after infection (Fig. 2b). In these cases, leaves were harvested before the appearance of symptoms.

RT-PCR analyses confirmed the expression of all constructs in the infiltrated leaves (Fig. 3b). Specific amplification products were obtained for each construct, whereas no signal was detected from mock-transformed plants (data not shown). Transiently expressed proteins were also found in all transformants except for the VP4Δ::VP7 construct (Fig. 3c). Molecular masses of the expressed proteins correspond to those predicted for the corresponding genes. The yield of expressed proteins ranged from 0.85 to 31.97 μg of recombinant protein per gram of fresh leaf tissue (Table 2). No evidence of degradation products was observed on the immunoblots indicating that the expressed proteins are stable in the plant system. Thus, most of the plant-optimized genes are expressed properly in the *Nicotiana benthamiana* system.

Expression of the VP7 construct in our transient expression conditions resulted in severe toxicity and low protein yield. In contrast, plants infiltrated with the VP4Δ and fljB constructs showed little bleaching and fewer necrotic lesions on leaves (Fig. 2b). Expression of these constructs was moderate with yields of 5 and 4.1 μg of recombinant protein per gram of fresh leaf tissue, respectively (Table 2). The recovered quantity of VP4Δ was comparable to that reported for the VP8* subunit transiently expressed in *Nicotiana benthamiana* leaves (4 μg/g of fresh leaf tissue) [28]. Compared to VP4Δ, the expression of VP7 was fivefold lower (0.85 μg/g of fresh leaf tissue). Previous studies reported that stable expression of VP7 in transgenic potatoes yielded 40 μg/g of tuber [29]. This discrepancy is probably due to the stable transformation or to the difference in plant system. However, our

Table 2 Production of recombinant proteins in *Nicotiana benthamiana* following *Agrobacterium*-mediated transient expression

Extracts	Transcript	Protein	MW (kDa)	Yield (μg/g)
Single constructs				
VP7	+	+	38.4	0.85
VP4Δ	+	+	38.5	5.0
fljB	+	+	53.2	4.1
Dimeric constructs				
VP7::VP4Δ	+	+	76.8	ND
VP4Δ::VP7	+	−	76.8	ND
VP7::fljB	+	+	90.9	31.97
VP4Δ::fljB	+	+	91.2	12.3

Transcript and protein accumulation were determined by RT-PCR and immunoblot analysis, respectively. (+) and (−) indicate presence or absence of signal. The proteins detected on SDS-PAGE gels (not shown) migrated to the predicted molecular weight of the various proteins. The yield of target protein in leaf extracts was determined by blot densitometry using calibrated protein standards, and is expressed as μg of recombinant protein per gram of fresh weight of agro-infiltrated leaves

ND not determined

system is rapid and efficient for most of the proteins tested, compared to the time-consuming generation of transgenic plants.

The most conserved domain of eubacterial flagellin, flg22, induces defense responses in various dicotyledonous plants and appears to behave as an elicitor of the Serk3/BAK1 pathway [26]. This pathway regulates the expression of various defense-related genes, which opened the way for the use of flagellins in strategies aiming to improve plant biotic stress. The development of transgenic plants that produce a flagellin has been reported only for rice [30]. This study reported that plants expressing the N1141 flagellin show enhanced disease resistance to *Magnaporthe grisea*, the causal agent of rice blast. Work is in progress in our laboratory to assess the potential of fljB to induce the expression of defense-related genes and improve resistance to biotic stress in transgenic *Arabidopsis thaliana* plants.

The assembly of epitopes was previously shown to improve the immune response [31]. Therefore, the selected VP7, VP4, and fljB epitopes were assembled in various antigen-antigen combinations. Recent studies in mice have shown that a fusion between the rotavirus VP8* domain and the amino-terminal end of a truncated VP2 (first 92 amino acids) induces a high titer of VP4-specific antibodies [32]. Unfortunately, the VP4Δ::VP7 antigen–antigen construct induced damage in infiltrated leaves and the chimeric protein was undetectable by immunoblot analysis (Fig. 3c). This prompted us to test if the reciprocal construct VP7::VP4Δ protein could be used. The data show that leaves of plants infiltrated with this construct display milder bleaching symptoms and accumulate a low level of protein (Fig. 3c; Table 2). The expression of fljB flagellin and VP::fljB fusions has no effect on plant growth. Plants infiltrated with the VP7::fljB and VP4Δ::fljB constructs demonstrate minimal damage, and express the proteins to relatively high levels (32 and 12 μg of recombinant protein per gram of fresh leaf tissue, respectively) (Table 2). These higher yields compared to single antigens could be due to the fusion of the VP antigens with the fljB fragment. Previous efforts to produce rotavirus antigens in plants have been reported [33–36]. However, none of these studies showed the expression of VP4Δ or fljB or the fusion of rotavirus antigens with fljB. The results presented here show that the plant transient system is efficient in expressing multiple combinations of pathogenic proteins which could be useful in studies aiming to determine the functions of viral proteins and in the development of diagnostic tests and vaccines [37].

Humoral Response in Mice

To examine the protein-specific antibody response in mice, the systemic immunogenicity of the plant-derived antigens was investigated in BALB/c mice by subcutaneous injection of the plant extracts. For our study, we selected single antigen constructs VP7 and VP4Δ because these proteins are known to induce the production of neutralizing antibodies. However, immunoblot analyses showed that immunization with VP7 or VP4Δ did not elicit the production of HRV-specific antibodies in mice (Fig. 4a). Results of indirect immunofluorescence and lymphoproliferation analyses (data not shown) were inconclusive, suggesting that the humoral and cellular responses in mice immunized with the HRV antigens are very weak and difficult to detect. This is probably due to the fact that the quantities of target recombinant proteins used for the

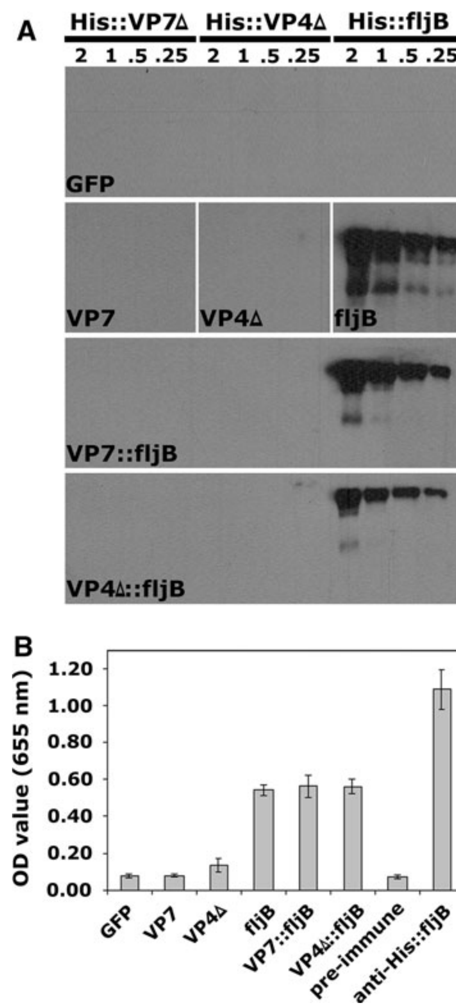

Fig. 4 Detection of antigen-specific antibodies in mice antisera. **a** His-tagged recombinant VP7Δ, VP4Δ, and fljB proteins (0.25–2 μg; indicated at the top) were analyzed by immunoblotting with antisera obtained from mice immunized with the various plant-expressed proteins as indicated on the panels. **b** Serum (1:10,000 dilution) fljB-specific IgGs were measured by indirect ELISA. The values are presented as mean ± SD. Mouse pre-immune serum and rabbit anti-His::fljB antibody were used has negative and positive controls, respectively

primary immunization and/or the number of booster injections were insufficient. In contrast, the immunoblot and ELISA analyses (Fig. 4a, b) confirmed that immunization with fljB, VP7::fljB or VP4Δ::fljB generated a strong and specific response against the fljB moiety. The accumulation of fljB-specific antibodies corroborates the high immunogenic potential of flagellins. *Salmonella*'s flagellin is well known PAMPs that activate innate immunity through the toll-like receptor TLR5 [38]. Immunization with doses as low as 4.1 μg of recombinant fljB protein induced the production of specific antibodies. The stimulation of a humoral response confirms the potential of using plants for the development of edible vaccines against *Salmonella typhimurium*. Interestingly, Vijay-Kumar et al. [39] showed that systemic administration of *Salmonella typhimurium* flagellin protected mice against chemical, bacterial, viral, and radiation challenge without any adverse effect. These observations, along with the fact that flagellin induces the expression of various genes with anti-apoptotic, antibacterial, and other cytoprotectant activities [39], suggest that a flagellin treatment could protect human populations against select dangers. The production of relatively safe immune activators such as flagellin will certainly be advantageous for these applications.

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