

# Expression of the VP40 antigen from the *Zaire ebolavirus* in tobacco plants

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## Abstract

**Main conclusion** The plant cell is able to produce the VP40 antigen from the *Zaire ebolavirus*, retaining the antigenicity and the ability to induce immune responses in BALB/c mice.

The recent Ebola outbreak evidenced the need for having vaccines approved for human use. Herein we report the expression of the VP40 antigen from the Ebola virus as an initial effort in the development of a plant-made vaccine that could offer the advantages of being cheap and scalable, which is proposed to overcome the rapid need for having vaccines to deal with future outbreaks. Tobacco plants were transformed by stable DNA integration into the nuclear genome using the CaMV35S promoter and a signal peptide to access the endoplasmic reticulum, reaching accumulation levels up to 2.6  $\mu\text{g g}^{-1}$  FW leaf tissues. The antigenicity of the plant-made VP40 antigen was evidenced by Western blot and an initial immunogenicity assessment

in test animals that revealed the induction of immune responses in BALB/c mice following three weekly oral or subcutaneous immunizations at very low doses (125 and 25 ng, respectively) without accessory adjuvants. Therefore, this plant-based vaccination prototype is proposed as an attractive platform for the production of vaccines in the fight against Ebola virus disease outbreaks.

**Keywords** Antigen delivery system · Ebola · Humoral response · Molecular pharming · Oral vaccine · Subunit vaccine

## Abbreviations

EVD	Ebola virus disease
EBOV	Ebola virus
GP	Glycoprotein
VP	Viral protein

## Introduction

The 2014–2015 Ebola virus disease (EVD) outbreak in West Africa (Liberia, Sierra Leone, Mali, Nigeria, and Senegal) had raised the alarms and evidenced the lack of substantial Ebola virus (EBOV) treatments, thus there is an important need for having scalable production platforms to generate EBOV vaccines. According to the World Health Organization (WHO), from March 2014 to March 2016, there were 28,646 infected people and 11,323 casualties (WHO 2015). The EBOV is an enveloped, negative sense, single-stranded RNA virus whose genus comprises five species: (1) *Sudan ebolavirus*; (2) *Zaire ebolavirus*; (3) *Côte d'Ivoire ebolavirus* (also known as *Ivory Coast ebolavirus*); (4) *Reston ebolavirus*, and (5) *Bundibugyo ebolavirus*. All of these species, with the exception of the

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*Reston ebolavirus*, have been shown to cause disease in humans (Kuhn et al. 2010; Marsh et al. 2011). The EBOV codes for seven structural proteins and three nonstructural proteins: soluble GP (sGP), secreted soluble GP (ssGP), and  $\Delta$ -peptide (Feldmann et al. 2006). The functions of the nonstructural proteins are unknown. Structural proteins include glycoprotein (GP), nucleoprotein (NP), RNA polymerase L, and viral proteins (VP24, VP30, VP35, and VP40). GP is responsible for binding to host cell surface receptors, membrane fusion, and viral entry. NP and VP30 along with VP35 and RNA polymerase L interact with the EBOV RNA genome to form the ribonucleocapsid complex (Feldmann et al. 2006; Noda et al. 2010). All the ribonucleocapsid complex-associated proteins are involved in transcription and replication of the genome, whereas the envelope-associated proteins (GP, VP24, and VP40) play a role in virion assembly, budding, and virus entry (Feldmann et al. 2006; Noda et al. 2010). The functions of VP24, VP35, and VP40 (to a lesser extent) include the suppression of interferon (IFN) signaling pathways in the host (Feldmann et al. 2006).

It is believed that humans initially acquire the virus from fruit bats belonging to the family *Pteropodidae*, which act as infected reservoirs (Leroy et al. 2009). However, no infectious EBOV or complete RNA genome has ever been isolated from fruit bats. In the history of the EVD, only the Tai Forest virus (TAFV) has been associated with chimpanzees. Outbreaks of the EVD are driven by human-to-human transmission through infected bodily fluids such as sweat, vomitus, blood, and urine. The EVD is characterized by non-specific initial symptoms such as sudden onset of fever, chills, and malaise accompanied by other signs such as myalgia, headache, nausea, abdominal pain, vomiting, and diarrhea (Bociaga-Jasik et al. 2014). The incubation period varies from 3 to 21 days (WHO 2014). More characteristic hemorrhagic symptoms such as melena, hematochezia, epistaxis, hematemesis, and bleeding from injection sites appear later in the course of the disease. Prolonged dehydration and development of multi-organ dysfunction lead to irreversible shock ending in death. Although a number of vaccines and drugs are currently under development, no licensed treatments for treatment of the EVD are available thus far. The VP40 antigen has been proposed as target in the development of subunit vaccines, which was supported by the evidence on correlates of protection described in immunized test animal models (Warfield et al. 2003). VP40 is considered an attractive target for vaccine development since it is a non-glycosylated protein that forms VLPs not requiring the presence of other proteins (e.g., GP1). VP40 self-assembly is mediated by the PTAP and PPXY motifs, which interact with the WW domains of the host proteins (Jasenosky et al. 2001, Noda et al. 2002; Freed 2002). Of particular interest are the consecutive series of studies by the

US Army medical research team, which demonstrated the potential of Ebola virus-like particles (VLPs) formed by GP/VP40 as an effective vaccination strategy against EVD. Three intramuscular (i.m.) immunizations with 10  $\mu$ g doses of Ebola VLPs, expressed in the mammalian cell line 293T without adjuvants, successfully protected mice against a lethal EBOV challenge (no yields were reported) (Warfield et al. 2003). In addition, when administered in combination with QS-21 as adjuvant, the VLPs were able to protect against a lethal EBOV challenge after two i.m. doses in mice (Warfield et al. 2005), whereas a single-dose of i.m. administered RIBI adjuvant protected guinea pigs (Swenson et al. 2005). The most outstanding stride thus far by this group comprises the protection of non-human primates against a lethal EBOV challenge when given in formulation with the RIBI adjuvant (Warfield et al. 2007). In another approach, VP40 has also been expressed in insect cells using a recombinant baculovirus system, which was able to elicit strong specific immune responses in mice after two i.m. 10  $\mu$ g doses (Sun et al. 2009). It is worth pointing out that the EVD vaccination approaches explored thus far are based on parenteral immunizations that often require antigen purification or the use of accessory adjuvants (Swenson et al. 2005; Warfield et al. 2005, 2007; Sun et al. 2009).

The use of plants in the production of vaccines is a relevant field with important advances achieved in the last decade. Plant-made vaccines have several advantages comprising the expression of high-quality recombinant proteins at low costs with a reduction of contamination risks with human pathogens (Hernández et al. 2014; De Martinis et al. 2016). Stable transformation at the nuclear level leads to lines that inherit the trait, but require long generation time (at least few months), with modest yields in the 0.02–0.5 mg per kg of fresh plant biomass range.

In contrast, transient expressions rely on temporal expression at the nuclear level in adult plants, with the subsequent biomass harvesting in the short term (within 2 weeks) (Govea-Alonso et al. 2014). Transient expression systems have been optimized to achieve high yields (up to 5 g per kg of fresh leaves), allowing the purification of the antigen for the production of parenteral formulations that will likely be the first to be commercialized (Pillet et al. 2016). Transient expression is predominantly restricted to *Nicotiana benthamiana* which is not suitable for oral administration, whereas antigens can be produced in edible plants via stable transformation. Given the differences in the glycosylation of plants with respect to mammals, the implementation of glycoengineering approaches has added an additional advantage to the molecular farming field (Loos and Steinkellner 2014).

In addition, plant-made vaccines offer the possibility of performing the oral delivery of formulations made with freeze-dried plant biomass, which avoids the costs derived

from purification and parenteral delivery. The current aim of the technology implies that a reduction in production and preservation costs could represent a real step forward to improve therapy coverage, especially in developing countries where humanitarian initiatives are needed to achieve global vaccination (Govea-Alonso et al. 2014). The potential of this application has been demonstrated through several clinical trials performed with a wide range of plant species expressing different antigens, most of them using raw plant material for oral immunization (Tacket 2009; Takeyama et al. 2015).

In the case of Ebola, only few biopharmaceuticals have been produced in plant systems. On one hand, plant-made mAbs have been produced in *N. benthamiana* as host through a conventional nuclear stable transformation expression system (Qiu et al. 2014). Remarkably this mAbs cocktail, ZMapp, currently constitutes the most promising therapy against Ebola. In fact, ZMapp was approved in 2014 for testing in infected people during the West Africa outbreak as an emergency action. Only two of the seven ZMapp-treated patients died (McCarthy 2014). Another promising plant-made biopharmaceutical against Ebola comprises GP1-6D8 IgG-Ebola immune complexes produced in *N. benthamiana* through the use of a geminiviral replicon system (Phoolcharoen et al. 2011).

Since plants have been extensively used as efficient, economic, and safe vaccine production platforms the aim of the present study is to express EBOV VP40 in *Nicotiana tabacum* assessing its immunogenicity in BALB/c mice.

## Materials and methods

### Molecular cloning and transgenic tobacco development

A synthetic gene, named VP40, was designed to encode the full-length VP40 protein from the *Zaire ebolavirus* strain Zaire 1995 (GenBank acc. no. AY354458.1) with the addition of a signal peptide (API: ATGGGGGCATCGAG GAGTGTTTCGATTGGCTTTCTTCTTGTTGTTTTGGT AGTATTAGCA GCCTTAGCTGAGGCA) from the *Physcomitrella patens* vacuolar aspartic proteinase, which is intended to increase the accumulation of the recombinant protein by translocation into the endomembrane system. The codon-optimized gene was defined and synthesized by GenScript (Piscataway, NJ, USA), and subsequently sub-cloned into the pBI121 binary vector using the restriction sites *Sma*I and *Sac*I by conventional procedures (Jefferson 1987). The positive recombinant clones were verified by PCR using VP40-specific primers (sense 5' ACCCGGGAT GAGCGGGTTATATTACCTAC 3'; antisense 5' GAGC TCGGATCCTTACTTCTC 3') and conventional

sequencing. DNA from a positive clone was electroporated into the *Agrobacterium tumefaciens* GV3101 strain according to the method described by Cangelosi et al. (1991).

Transgenic tobacco (*N. tabacum* cv. Petit Havana SR1) plants were generated following the method described by Horsch et al. (1985). After co-cultivation, the explants were cultivated on selection medium. Cultures were maintained in a controlled environment chamber (25 °C) under a 16-h light/8-h dark photoperiod and transferred to fresh medium biweekly. After generation of shoots, those attaining 1 cm in length were excised and placed in rooting medium. Rooted plantlets were subsequently acclimatized in soil and grown in a greenhouse.

### Transgene detection and determination of copy number

Total DNA was isolated from leaves of both putative transformants and wild-type plants according to Dellaporta et al. (1983). A 25- $\mu$ L PCR reaction mixture was prepared to amplify the *VP40* gene using the above described primers. Cycling conditions and amplicon detection were performed as described elsewhere (Monreal-Escalante et al. 2016). The positive control consisted in a reaction containing 10 ng of the pBI-VP40 vector as template, whereas the negative control consisted in a reaction containing 100 ng of DNA from a WT plant.

A real-time PCR (q-PCR) was performed to assess the number of transgene copies inserted in the plant genome using a previously described method (Salazar-Gonzalez et al. 2014). The primer pairs for the *VP40* gene (forward 5' TTACCTACTGCTCCTCCTGAA; reverse 5' GTATTGCT GTTGCCACCTCTA), as a part of the transgene construction, and the *Tob103* gene coding for actin (forward 5' CAACCACCGAAGACTGAGAAG; reverse 5' GGAG-GATTAGGTGTGTGTAG), as a single copy reference gene were used. PCR was carried out in 48-well reaction plates (Applied Biosystems, Foster City, CA, USA) using the SUPER SYBR Mix (Applied Biosystems) with primers to a final concentration of 200 nM in 10  $\mu$ L of total volume reaction. PCR was run using a real-time system (StepOne™, Applied Biosystems) under the following cycling conditions: 95 °C for 10 min (initial denaturation), 40 cycles at 95 °C for 15 s, and 60 °C for 60 s for annealing and extension.

### Hyperimmune sera production

The animals used in this study were maintained under standard laboratory conditions with free access to food and water following procedures established by the Federal Regulation for Animal Experimentation and Care

(SAGARPA, NOM-062-ZOO-1999, México), approved by the Institutional Animal Care and Use Committee. Thirteen-week-old female BALB/c mice were immunized on day 1 in the rear footpad with 5 µg of recombinant VP40 protein (supplied by Sino Biological Inc., Beijing, China; and purified from recombinant *E. coli* cultures by Immobilized Metal Affinity Chromatography) emulsified in 20 µL of complete Freund's adjuvant (CFA). Three subsequent doses were intraperitoneally administered on days 8, 15, and 22, consisting of 5 µg of VP40 emulsified in one volume of incomplete Freund's adjuvant (IFA). Mice were bled on day 29 to measure antibody titers. The animals were subsequently killed to collect sera.

### VP40 immunodetection

Protein extracts were obtained by grinding ~300 mg of fresh leaf tissues in the presence of 200 µL of cold protein extraction buffer (750 mM Tris-HCl, pH 8, 15% sucrose, 100 mM β-mercaptoethanol, and 1 mM PMSF). ELISA analysis was performed using plates coated overnight at 4 °C with 50 µL of protein extracts per well, diluted in carbonate buffer (0.2 M, pH 9.6). Three washes with PBST were performed between each step. A subsequent blocking step was performed using 5% fat-free dry milk solution for 2 h at 25 °C. A further labeling with a 1:1500 dilution of the anti-VP40 serum described above was performed overnight at 4 °C. A rabbit horseradish peroxidase-conjugated anti-mouse IgG (1:2000 dilution; Sigma, <http://www.sigmaaldrich.com>) served as the secondary antibody after 2 h of incubation at 25 °C. An ABTS solution (0.6 mM 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid), 0.1 M citric acid, pH 4.35, 1 mM H<sub>2</sub>O<sub>2</sub>) was used for immunodetection. After incubating 30 min at 25 °C, OD values at 405 nm were recorded using an iMark™ microplate reader (Bio-Rad, Hercules, CA, USA). A standard curve was constructed using pure VP40 (Sino Biological, Inc) to estimate the expression levels in samples from transgenic plants.

For Western blot analysis, the protein extracts were previously adjusted to equal amounts of total soluble protein (10 µg) using the Bradford's method. Protein samples were mixed with one volume of 2× Laemmli reducing protein loading buffer and denatured by boiling for 5 min at 95 °C. Debris were eliminated by centrifugation at 16,000g for 10 min. SDS-PAGE analysis was performed using 4–12% acrylamide gels under denaturing conditions; the resulting gel was blotted onto a BioTrace PVDF membrane (Pall Corporation, <http://www.pall.com>). After blocking in PBST plus 5% fat-free milk (Carnation, Nestle, <http://www.nestle.com>), blots were incubated overnight with the anti-sera against VP40 obtained in the present study at a 1:1500 dilution. A rabbit horseradish peroxidase-

conjugated anti-mouse IgG antibody (1:2000 dilution; Sigma, <http://www.sigmaaldrich.com>) was added and incubation for 2 h at room temperature was performed. Antibody binding was detected by incubation with SuperSignal West Dura solution (Thermo Scientific, <https://www.thermofisher.com>) following the instructions from the manufacturer. Signal detection was conducted by exposing an X-ray film using standard developer and fixer solutions.

### Immunogenicity assessment

Immunogenicity of tobacco biomass containing the plant-made VP40 was assessed in 13-week-old female BALB/c mice with 25 g of body weight. A total of four test mice groups ( $n = 4$ ) received one of the following treatments: transgenic tobacco s.c. administered, WT tobacco s.c. administered, transgenic tobacco orally administered with the aid of an intragastric probe, or WT tobacco orally administered with the aid of an intragastric probe. The doses were prepared milling fresh leaf tissue from adult plants of T0 lines in PBS at a 1:1 ratio. The subcutaneous doses were derived from 10 mg of plant tissue, whereas the oral doses from 50 mg of leaf tissue. The groups were subjected to four weekly immunizations (on days 1, 8, 15, and 22). On day 29, sera and intestinal washes samples were collected as previously described (Monreal-Escalante et al. 2016). Sera and intestinal washes were stored at -70 °C until further use in antibody measurements.

ELISA analyses were performed to assess the presence of VP40-specific antibodies in mice samples. ELISA plates were coated overnight at 4 °C with a pure recombinant VP40 protein (250 ng per well; Sino Biological, Inc.) and subsequently blocked with 5% fat-free dry milk for 2 h at 25 °C. Plates were incubated overnight at 4 °C with serial dilutions of sera from mice (1:10 to 1:160 dilutions). Mouse horseradish peroxidase-conjugated anti-mouse IgG, IgM or IgA (Sigma) were used as secondary antibodies at a 1:2000 dilution and the plates were further incubated for 1 h at 25 °C. Antibody binding was detected as described above. Data derived from ELISA studies were analyzed by one-way ANOVA (Statistica 2.7 software). A *P* value less than 0.05 was considered statistically significant.

## Results

The pBI121 binary vector was used to develop a plant expression vector for the V40 protein. This binary vector possesses *uidA* as a reporter gene and the kanamycin resistance gene *nptII* as marker genes under the control of NOS and CaMV35S promoters, respectively. Since the synthetic gene was provided into the pUC57 cloning

vector, a subcloning step was performed to substitute the reporter gene at pBI121 by the gene of interest using the restriction sites *Sma*I and *Sac*I. The binary vector was successfully assembled as confirmed by restriction profiles and sequencing (Fig. 1a), and subsequently transferred to *A. tumefaciens*. Following the Agrobacterium-mediated transformation procedure, several candidate tobacco lines were isolated in selective media. A total of seven putative transformed lines were successfully regenerated and acclimatized in soil. PCR analysis was performed to these putative transformants. Figure 1b shows the presence of  $\approx$  1100 bp amplicons in all the samples from the putative transgenic lines, which is consistent with the expected size and the amplicon yielded by the positive control (pBI-VP40). No signal was observed for the WT DNA sample.

The transgene copy number was next determined by qPCR. To construct standard curves, the target or endogenous genes were amplified from a randomly chosen T0 transgenic line (L6) in duplicates of a twofold serial dilution of seven genomic DNA concentrations in the 3.125–400 ng/ $\mu$ L range. To determine the transgene copy number, PCR was performed in triplicate with  $\sim$  50 ng of DNA from transgenic lines L3, L4, L5, L6, or WT plants and  $C_t$  values for both, the transgene and the endogenous gene, were determined under the same auto baseline and threshold. The results were analyzed using the StepOne™ Software v2.1

The transgene copy number was calculated using the following equation:

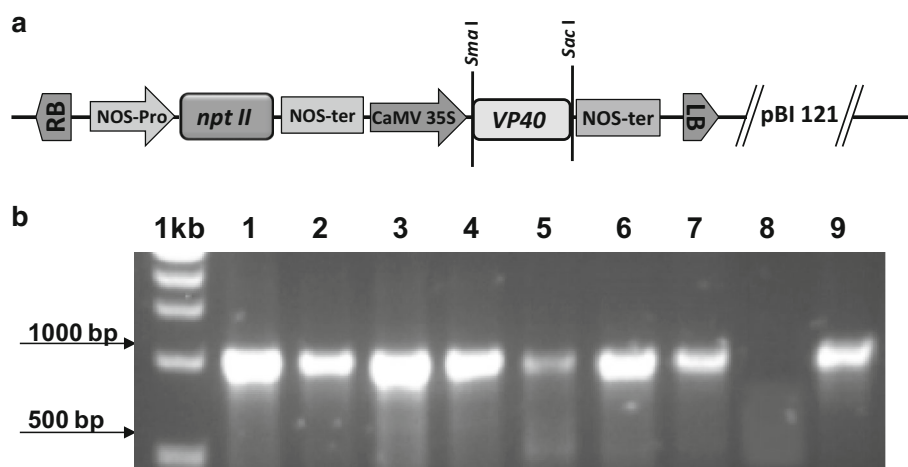
$$X_0/R_0 = 10^{[(C_{t,X}-IX)/SX]-[(C_{t,R}-IR)/SR]}$$

where  $R_0$  is the initial amount of reference copies,  $X_0$  is the initial amount of copies of the target gene, IX and IR are

intercepts of the relative standard curves of target and reference genes, respectively; SX and SR are the slopes for both genes, and  $C_{t,X}$  and  $C_{t,R}$  are the  $C_t$  values of the target and reference genes, respectively. The equation was applied on the basis that if the copy number of the reference gene ( $R_0$ ) is known, the copy number of the target gene ( $X_0$ ) can be deduced from the values for  $C_{t,X}$ ,  $C_{t,R}$ , SX, SR, IX, and IR obtained in the tested sample. Analysis of the four selected transgenic lines (L3–L6) revealed three copies of transgene for lines L3, L5, and L6, whereas line L4 had 14 copies.

The expression of the recombinant plant-made VP40 protein was analyzed next. First, an ELISA study was performed in which higher OD values were observed in protein samples from the transgenic lines expressing VP40 (mean values in the 0.33–0.57 range) when compared to those recorded in the protein extract from the WT line (mean 0.05) (Fig. 2a). Quantification of the VP40 levels using the standard curve revealed values in the range from 0.6 to 2.6  $\mu$ g g<sup>-1</sup> FW, with line L5 having the maximum VP40 accumulation. A Western blot using specific VP40 serum was performed with protein extracts from transgenic and WT lines. A  $\approx$  38 kDa reactive band was observed in four test lines (L3, L4, L5, and L6), matching the theoretical molecular weight for VP40 (Fig. 2b).

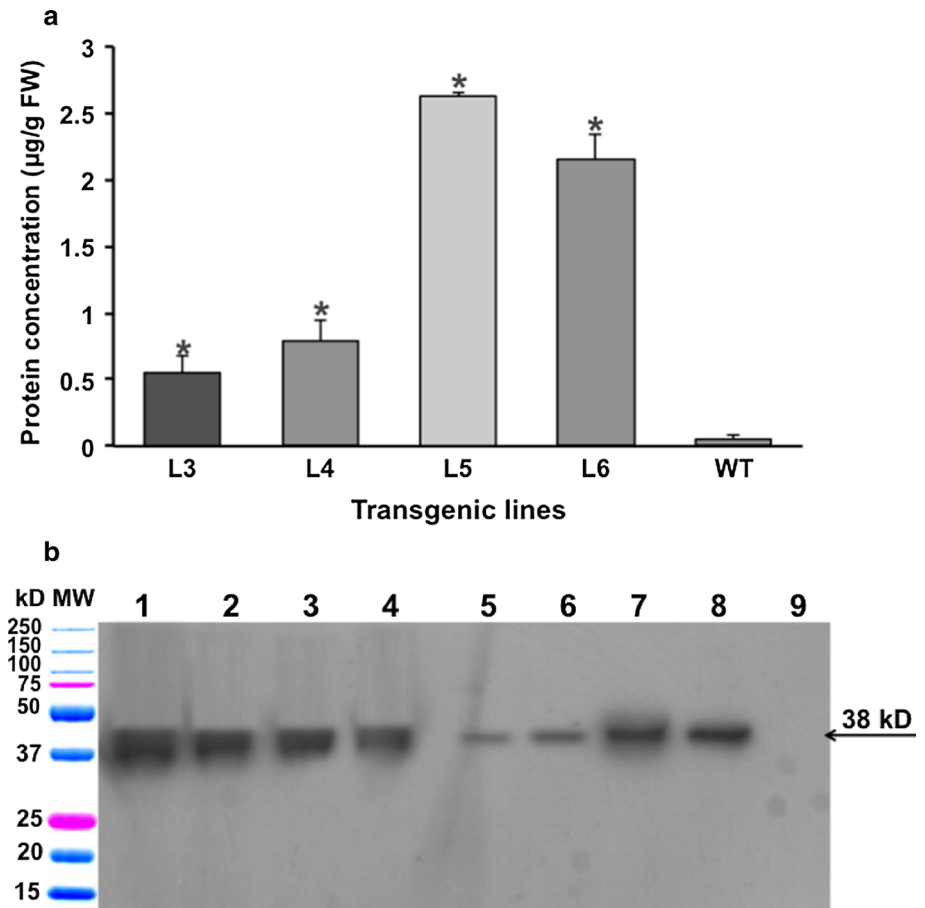
Based on the VP40 yields, line L5 was selected to assess the immunogenicity of the VP40 protein in BALB/c mice. After a scheme comprising three weekly doses administered either subcutaneously (dose 25 ng) or orally (dose 125 ng), ELISA analyses were performed to assess the presence of VP40-specific antibodies. Specific IgG responses were developed in both s.c. and orally immunized mice. The humoral response showed a sustained



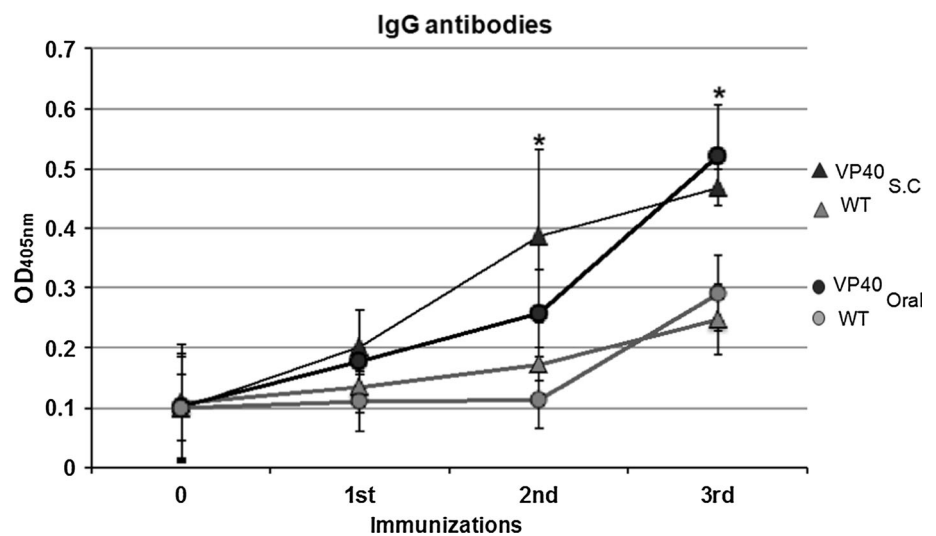
**Fig. 1** **a** Physical map of the binary vector used to express VP40 in plants. A binary vector having the pBI121 vector as backbone was constructed by cloning a synthetic VP40 coding sequence at the *Sma*I and *Sac*I sites present in the pBI121 plasmid. The VP40 gene was optimized according to codon usage in plants. **b** PCR analysis of

putative transgenic tobacco lines. Total DNA preparations were used to amplify the VP40 gene. Lane 1 kb molecular weight marker, lanes 1–7 DNA of transgenic plants lines L1–L7, lane 8 DNA of WT plant, lane 9 positive control (pBI-VP40 DNA, 10 ng)

**Fig. 2** Immunodetection and quantification of the tobacco-made VP40. **a** ELISA analysis of total protein extracts from transgenic and WT tobacco lines. Labeling was performed with anti-VP40 hyperimmune sera; the positive control consists of 100 ng of pure *E. coli*-made VP40. The asterisk denotes significant difference versus the WT ( $P < 0.05$ ). **b** Immunodetection was performed in total soluble protein extracts subjected to blotting and a subsequent labeling with an anti-VP40 serum. A densitometry analysis was conducted to determine the levels of the VP40 antigen. Lane *MWM* molecular weight marker, lane 1–4 VP40 standards (250, 125, 65, and 50 ng), lanes 5–8 protein extracts from lines L2–L5, lane 9 protein extract from a WT plant



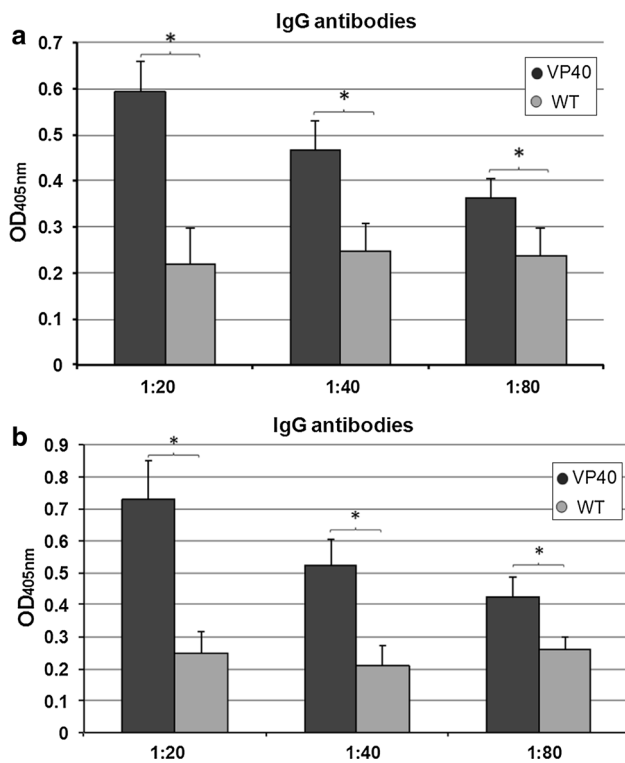
**Fig. 3** Anti-VP40 IgG levels in sera from mice immunized with the plant-made VP40. Mice groups were immunized orally or subcutaneously with the plant-made VP40 or WT tobacco in a three-weekly dose immunization scheme. IgG levels were determined by ELISA using a 1:40 dilution of sera samples, and coating plates with recombinant VP40. The asterisk denotes significant difference versus the WT ( $P < 0.05$ )



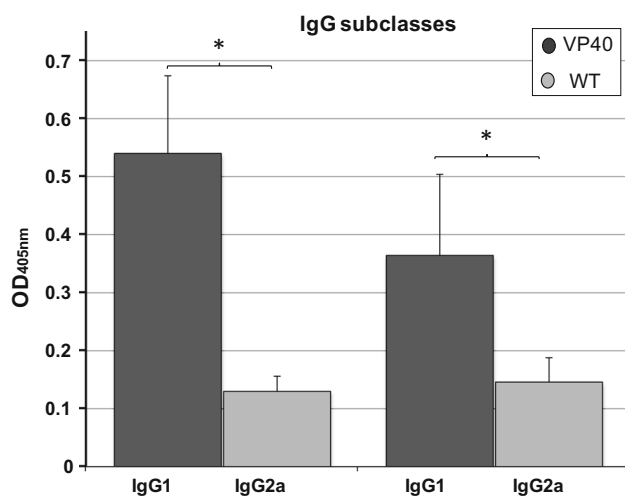
increase along the immunization scheme (Fig. 3). After three immunizations, both orally and s.c. immunized mice developed IgG serum responses at 1:80 titers (Fig. 4). The IgG subclasses were determined revealing higher levels of the IgG1 subclass, which indicates a predominant Th2 immune response (Fig. 5). The IgM measurements

indicated that seroconversion occurred after priming with a significant and sustained decrease at subsequent time points (Fig. 6).

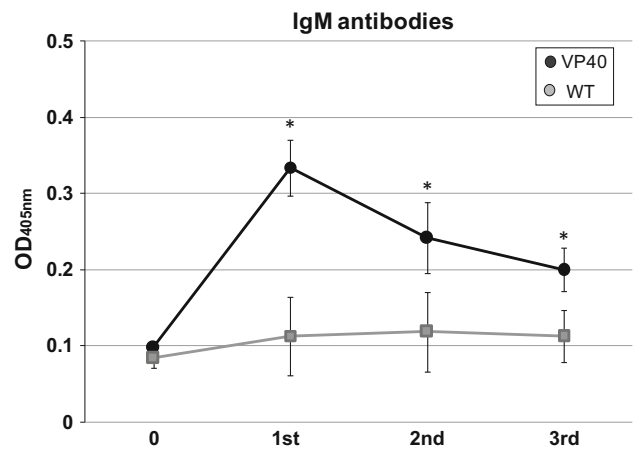
Intestinal washes were analyzed to determine the presence of mucosal IgA responses. Statistically significant IgA levels were detected in mice groups fed with tobacco-made



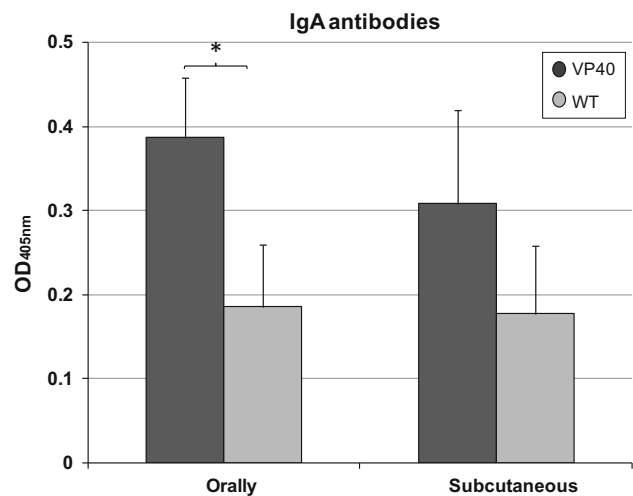
**Fig. 4** Determination of IgG titers in sera from mice immunized with the plant-made VP40. Mice groups were immunized orally or subcutaneously with the plant-made VP40 or WT tobacco in a three-weekly dose immunization scheme. IgG levels were determined by ELISA coating plates with recombinant VP40. **a** Titers in the orally immunized group. **b** Titers in the subcutaneously immunized group. The *asterisk* denotes significant difference versus the WT ( $P < 0.05$ )



**Fig. 5** Determination of anti-VP40 IgG subclasses levels in sera from mice immunized with the plant-made VP40. Mice groups were immunized subcutaneously with the plant-made VP40 or WT tobacco in a three-weekly dose immunization scheme. IgG subclasses levels were determined by ELISA coating plates with recombinant VP40. The *asterisk* denotes significant difference versus the WT ( $P < 0.05$ )



**Fig. 6** Determination of anti-VP40 IgM levels in sera from mice immunized with the plant-made VP40. Mice groups were immunized subcutaneously with the plant-made VP40 or WT tobacco in a three-weekly dose immunization scheme. IgM levels were determined by ELISA coating plates with recombinant VP40. The *asterisk* denotes significant difference versus the WT ( $P < 0.05$ )



**Fig. 7** Determination of anti-VP40 IgA in intestines from mice immunized with the plant-made VP40. Mice groups were immunized orally or subcutaneously with the plant-made VP40 or WT tobacco in a three-weekly dose immunization scheme. IgA levels were determined by ELISA coating plates with recombinant VP40. The *asterisk* denotes significant difference versus the WT ( $P < 0.05$ )

VP40, whereas no significant IgA responses were found in the s.c. immunized group (Fig. 7).

### Discussion

In the present study, the expression of VP40 in the plant cell was explored to open the path for developing new plant-based vaccination prototypes. The tobacco-derived VP40 was expressed at levels up to  $2.6 \mu\text{g g}^{-1}$  FW.

Although the accumulation levels depend on several factors, it can be hypothesized that the levels of VP40 in tobacco tissues are influenced by the ER signal peptide, which directs the VP40 protein to the secretory route. The lowest expression level observed was  $0.5 \mu\text{g g}^{-1}$  FW. The variation in the accumulation levels of VP40 among the tobacco-expressing lines could be associated to the several factors, such as the difference in the transgene copy number and insertion sites imposed by the non-specific and non-homologous random recombination induced by *A. tumefaciens* (Kim et al. 2007). Lines with low yields could be a consequence of transgene insertion in a non-transcriptional active chromosomal region due to high methylation or condensation degree, whereas higher expressing lines could be a consequence of transgene insertion in transcriptional active regions of the genome. In our case, the low VP40 yields observed in line L4 could be associated to the fact that it possesses 14 transgene copies, which could mediate posttranscriptional gene silencing. This high copy number of transgenes inserted in plant genomes through *Agrobacterium* transformation has been reported before (Ingham et al. 2001). In contrast, the lines having fewer transgene insertions (lines L5 and L6) showed higher VP40 yields ( $2.62$  and  $2.14 \mu\text{g g}^{-1}$  FW, respectively), with the exception of L3 ( $0.55 \mu\text{g g}^{-1}$  FW), in which transgene insertion could have occurred in genomic regions with low transcriptional activity.

Although VP40 production has been reported in other systems (e.g., 293T, Warfield et al. 2003), the lack of reports on the yields of VP40 achieved in recombinant systems limits a comparison with the tobacco lines generated in this study. Nonetheless, the yields observed in the present study for VP40 using tobacco are in the range for other biopharmaceuticals expressed in this crop (Chia et al. 2011). Since the VP40 protein was targeted into the secretory pathway and the endoplasmic reticulum is considered to have a low proteolytic activity, the observed overall yields could be attributed to protein degradation reduction in the cytosol (Benchabane et al. 2008).

Moreover, the tobacco-made VP40 retained the antigenic determinants as evidenced by immunoblot analysis using a specific anti-serum. Therefore, the evidence that the plant cell has the capacity to synthesize VP40 with no apparent toxicity or phenotypic alterations represents a step forward for the field. Thus far, the expression of EBOV antigens reported in plants is reduced to the work by Phoolcharoen et al. (2011) where immune complexes targeting GP1 were expressed in a transient expression system. The yields achieved in the mentioned study were  $50 \mu\text{g g}^{-1}$  FW leaf tissues. In contrast to the approach of Phoolcharoen et al. (2011), the stable transformed lines generated in the present study will facilitate the production of seed stocks to validate the batch-to-batch variability.

Our proposed system offers higher safety since the pathogen is not handled during the process and the plant cell does not act as host for human pathogens.

In terms of immunogenic activity, oral and s.c. administrations of VP40 encapsulated in the plant cell were explored to elicit humoral responses in BALB/c mice, observing that both immunization routes induced specific humoral responses with titers of 1:80. Since in previous studies a 1:20–1:160 titer range led to protective effects when a vaccine formulated with virus-like particles (VLPs) formed with GP1 and VP40 was used (Warfield et al. 2007), the titers achieved with the tobacco-made VP40 can be considered promising. The higher humoral response induced by low oral antigen doses (125 ng) lacking accessory adjuvants is of special interest. Previous studies using low doses (100 ng) of the small surface antigen of hepatitis B virus encapsulated in lettuce tissues have reported an immunogenic activity that enabled the induction of humoral responses at the nominally protective level (Pniewski et al. 2011). Another report of interest is related to the use of a low dose of an antigen expressed and delivered by tobacco cells following an oral immunization scheme comprising four weekly doses of 200 ng of antigen with a subsequent boost 1 week later using 400 ng of purified *E. coli* recombinant protein emulsified in Incomplete Freund's Adjuvant. Interestingly, specific immune response against *Toxoplasma gondii* and immunoprotection in terms of the number of developed cysts were achieved.

The induction of humoral response by the oral route using the complete plant biomass results of special interest since oral vaccination is the most advantageous immunization strategy in the developing world. This concept has been proven by several groups and the complex matrix of the plant cells has been proposed to effectively encapsulate antigens, which accounts for the immunogenicity of the oral vaccine formulation due to the presence of the cell wall, endomembrane systems, and several biopolymers (Rosales-Mendoza and Salazar-González 2014). Since the concept of delivering the functional immunogenic VP40 by the oral route has been generated in the present study, further studies optimizing plant-based oral vaccines targeting VP40 are justified. In addition, the use of edible crops to express VP40 will also be critical to advance in the development of oral vaccines with GRAS plant materials.

In the case of immunization by the s.c. route, significant responses were induced by the plant-made VP40. This preliminary evaluation of the plant-made VP40 will be followed by studies using the purified antigen to develop parenteral formulations.

Due to the natural tolerance to ingested antigens during normal dietary consumption, mucosal adjuvants have been developed to enhance immune responses for orally delivered vaccines looking to reduce the amount of antigen



required to elicit robust immune responses while ensuring the elicitation of appropriate types of immune responses (Lugade et al. 2010). Our VP40 vaccine elicited an immune response in mice without the inclusion of any mucosal adjuvant, such as the B subunit of cholera toxin (Nawar et al. 2005; Connell 2007). The observed immune polarization, a Th2 response, is convenient since the humoral response has been strongly associated to immunoprotection against EBOV (Becquart et al. 2014).

In conclusion, the detection of the antigenic and immunogenic VP40 in the plant cells represents a step forward in the development of plant-made vaccines targeting EBOV and opens perspectives for a detailed pre-clinic evaluation of this vaccine candidate.

**Author contribution statement** AARV and EME performed most of experiments. CA and SRM carried out the experimental design, data analysis and wrote the manuscript. EME and JASG participated in test mice experiments. BBH contributed to the data analysis. All authors discussed the results, read and approved the final version of the manuscript.

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