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ORIGINAL PAPER

An Env-derived multi-epitope HIV chimeric protein produced in the moss *Physcomitrella patens* is immunogenic in mice

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Abstract
Key message **The first report on the recombinant production of a candidate vaccine in the moss system.**
Abstract The need for economical and efficient platforms for vaccine production demands the exploration of emerging host organisms. In this study, the production of an antigenic protein is reported employing the moss *Physcomitrella patens* as an expression host. A multi-epitope protein from the Human Immunodeficiency Virus (HIV) based on epitopes from gp120 and gp41 was designed as a candidate subunit vaccine and named poly-HIV. Transgenic moss plants were generated carrying the corresponding *poly-HIV* transgene under a novel moss promoter and subsequently seven positive lines were confirmed by PCR. The poly-HIV protein accumulated up to 3.7 μg g^{-1} fresh weight in protonema cultures. Antigenic and immunogenic properties of the moss-produced recombinant poly-HIV are evidenced by Western blots and by mice immunization assays. The elicitation of specific antibodies in mice was observed, reflecting the immunogenic potential of this moss-derived HIV antigen. This is the first report on the production of a potential vaccine in the moss system and opens the avenue for glycoengineering approaches for the production of HIV human-like glycosylated antigens as well as other vaccine prototypes under GMP conditions in moss bioreactors.

Keywords HIV · Molecular farming · *Physcomitrella patens* · Plant-made pharmaceutical · Recombinant vaccine

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Introduction

Infections with the HI virus continue to be a major public health issue. The World Health Organization estimates that globally 35 million people lived with HIV infections in 2013, including 3.2 million children under 15 years, and 1.5 million people died of AIDS in 2013. Even though 2.5 million new infections are 22 % less than what was reported for the year 2001, an effective prophylactic HIV vaccine remains the best long-term strategy for preventing HIV/AIDS (WHO 2014). The development of a safe, effective, easy to administer and affordable HIV vaccine is urgently needed. However, one of the main challenges in the design of an effective vaccine for HIV consists of eliciting potent, broad and long-lasting cellular (CTLs) and humoral immune responses (Excler et al. 2014).

A widely used approach for developing HIV vaccine candidates employs recombinant DNA technology (Dong et al. 2005; Girard et al. 2006; Graham et al. 2010; Haynes et al. 2006; White and Meng 2012; Zolla-Pazner et al. 2008). Potential HIV vaccines are multi-epitope chimeric proteins that contain selected protective epitopes in a single antigen, while excluding the non-protective epitopes. The HI viral envelope (Env) proteins gp120 and gp41 are considered as the main targets for the development of broadly neutralizing antibodies due to their key interaction with the molecule CD4 and its co-receptor on CD4+ T lymphocytes (Mascola et al. 1996; Stott et al. 1998). The design of multi-epitope chimeric proteins has been proposed as one viable immunization approach for eliciting neutralizing antibodies targeting Env (Forsell et al. 2009; Rosales-Mendoza et al. 2012).

Among the multi-epitope HIV proteins reported so far, a candidate named C4(V3)6 consists of the fourth conserved domain from gp120 (C4) and six tandem repeats of a B cell epitope from the variable region V3 (Govea-Alonso et al. 2013). The C4 domain is an important part of the HIV CD4 binding site and contains T-helper and cytotoxic T lymphocyte epitopes. Also, it has been suggested that C4 exerts adjuvant effects associated to its role in CD4 binding (Patterson et al. 2001). Moreover, when used as a C4V3 chimeric arrangement, these regions have been able to trigger neutralizing responses against HIV-1 primary isolates (Liao et al. 2000; Haynes et al. 2006; Krachmarov et al. 2001; Letvin et al. 2001). Previous reports have shown that plant-derived proteins carrying the C4 and V3 sequences are orally immunogenic without the need of co-administered adjuvants (Govea-Alonso et al. 2013; Rubio-Infante et al. 2012).

The molecular farming field has gained industry interest as an attractive alternative for the production of vaccines using different expression hosts and strategies. In this arena, the moss *Physcomitrella patens* has been employed for the production of antibodies with superior lytic potential (Schuster et al. 2007) and other biopharmaceuticals such as Erythropoietin (Parsons et al. 2012; Weise et al. 2007), human Complement Factor H (Buettner-Mainik et al. 2011), human Vascular Endothelial Growth Factor VEGF (Baur et al. 2005) and human α-galactosidase, which is in clinical trials (www.greenovation.com). The distinctive features of this host are: it can be grown in contained bioreactor cultures (Hohe et al. 2002) up to 500L batches under full GMP conditions for pharmaceutical production, and it can be genetically modified by gene targeting due to its yeast-like efficient homologous recombination (Decker and Reski 2012). Precise genome alterations by gene targeting (Hohe et al. 2004) have been employed for the glycoengineering of production lines based on deletion and/or heterologous expression of specific glycosyltransferase genes (Koprivova et al. 2004; Huether et al. 2005; Parsons et al. 2012, 2013). This resulted in production lines devoid of any possibly adverse protein glycosylation patterns (reviewed in Decker et al. 2014). Moreover, key components of the mammalian transcription, translation and secretion machineries are functional in moss (Gitzinger et al. 2009) as are tunable synthetic promoters for transgene expression (Kubo et al. 2013; Müller et al. 2014). Despite the wide variety of successfully produced recombinant biopharmaceuticals in moss, its suitability for the production of new vaccines remains unexplored, but has been suggested recently (Rosales-Mendoza et al. 2014a).

Here, we report on the successful production of an Env-based HIV multi-epitope protein (poly-HIV) in transgenic moss lines and its functionality to elicit an immune response in mice.

Materials and methods

Protein design

The multi-epitope protein poly-HIV design comprises a plant secretion signal peptide and a set of epitopes, from either gp120 or gp41, in the following order: (i) C4, (ii) the V3 loop corresponding to a consensus sequence reported by Haynes et al. (2006), and (iii) four variants of the ELDKWA epitope, which is a highly conserved region recognized by broadly neutralizing antibodies (Dong et al. 2005; White and Meng 2012; Table 1). Glycine–Alanine linkers were included between each component. To facilitate the detection of the chimeric protein, a 6xHis-tag was included at the C-terminus. A synthetic gene coding for the poly-HIV protein was obtained from GenScript (Piscataway, NJ, USA), provided in the pUC57 cloning vector.

Table 1 Elements included in the moss-derived poly-HIV protein

Component	Sequences
Signal peptide	MAKLVFSLCFLLFSGCCFACMP
C4 from gp120	KQIINMWQEVGKAMYA
V3 from gp120	RPNNNTRKSIHIGPGRAFYATE
ELDKWA (5X) from gp41	ELDKWA
	KLDQWA
	ALDSWN
	ALDKWD
	ELDKWA

Construction of the expression vector

To obtain a poly-HIV expression vector that is functional in moss, the *poly-HIV* gene was subcloned into the pCA200P-35ST vector to obtain the pCA200P-HIV vector, where the poly-HIV is under the control of the *P. patens* carbonic anhydrase (Pp1s43_118V6.1, Zimmer et al. 2013) promoter and 5′ UTR (CA200P) spanning positions 970,129–970,999 in scaffold 43. An additional expression cassette in the vector allows for the expression of the *hpt* gene as a gene marker conferring hygromycin resistance. The subcloning step was mediated by the restriction sites *Sal*I and *Kpn*I. Selection of a positive clone for the construct was based on restriction profiles and conventional sequencing. The region comprising the *poly-HIV* and *hpt* expression cassettes was released from the pCA200P-HIV construct by restriction digest with *Eco*RI and *Hind*III and used for moss transformation.

Establishment of transgenic moss lines

The moss *Physcomitrella patens* (Hedw.) B.S. was cultivated under standard conditions in liquid or on solid Knop medium as described previously (Frank et al. 2005). Suspension cultures of WT and transgenic moss lines were subcultured in parallel weekly by disruption of the tissue with an Ultraturrax (IKA, Staufen, Germany) with a rotational speed of 19,000 rpm/min during 1 min and a subsequent transfer into fresh Knop medium (Reski and Abel, 1985). Protoplast isolation and polyethylene glycol-mediated transfection with linearized DNA constructs were performed as previously described (Frank et al. 2005). For selection of transformants, Knop media were supplemented with 12.5 μg/mL hygromycin (Sigma-Aldrich, Steinheim, Germany).

The screening of transformants was performed by direct PCR analysis of genomic DNA following the protocol of Schween et al. (2002). Briefly, small pieces of gametophore tissue were resuspended in 30 μl of PCR Buffer 1X (Genaxxon Bioscience, Ulm, Germany) containing three glass beads and subjected to vortexing for 1 min. Samples were subsequently incubated for 15 min at 45 °C and centrifuged at 20,000×*g* for 1 min. Samples were frozen at −20 °C for several hours and then heated at 45 °C for 30 min. Supernatants from these extracts (5 μL) were used as a template for PCR using the KAPA Plant PCR Kit (Kapa Biosystems, Wilmington, MA, USA) containing 10 μM of the primers specific for the *poly-HIV* gene (HIV-F, 5′-GGCGGGAGATGCAATAGGTCA-3′ and HIV-R, 5′-GGTAAATAGCTGCGCCGATGGTT-3′). A negative control using WT DNA and a positive control consisting of the pCA200P-HIV plasmid were run in parallel. In total, seven transgenic moss lines were identified and deposited in the International Moss Stock Center (IMSC, www.moss-stock-center.org) with the following accession numbers (IMSC No. 40745–No. 40751).

Detection of the poly-HIV protein

Transgenic moss plants were cultured as described above. Total soluble protein extracts from transgenic and WT protonemata were analyzed by immunoblot analysis. About 30 mg of moss tissue was disrupted in a mortar and resuspended in 200 μl of a buffer containing 750 mM Tris–HCl pH 8.0, 15 % sucrose (w/v), 100 mM β-mercaptoethanol and 1 mM phenylmethylsulfonyl fluoride (PMSF) (Franklin et al. 2002). Samples were prepared for electrophoresis under reducing conditions by adding 200 μL of loading buffer containing 200 mM Dithiothreitol (DTT) and boiling for 5 min. After centrifugation at 13,000 rpm for 10 min, 25 μL of the supernatant was resolved by SDS-PAGE in a 12 % acrylamide gel at 150 V for 1.5 h, blotted onto PVDF membranes in a wet electroblotting system for 1 h and subsequently blocked overnight by incubation in PBST plus 5 % non-fat milk. Primary labeling was performed at room temperature for 2 h using either a mouse anti-His or an anti-C4(V3)6 hyperimmune serum (1:200 dilution). An anti-mouse antibody coupled to peroxidase was used as secondary antibody (Sigma-Aldrich, 1:5000 dilutions). Signal detection was performed using the Pierce ECL Western Blotting Substrate (Thermo Scientific, Massachusetts, USA) following the manufacturer's instructions and an X-ray film under standard procedures.

Antigenicity evaluation

To evaluate antigenicity, an ELISA analysis was conducted using a hyperimmune serum raised against a synthetic peptide containing tandem repeats of the ELDWKA epitope or the C4(V3)6, a previously reported HIV antigen carrying sequences from the C4 and V3 gp120 domains of distinct HIV isolates (Govea-Alonso et al. 2013). To obtain

hyperimmune sera, mice were primed by a single dose administration in the paw of 10 µg of antigen emulsified into 10 µl of Freunds complete adjuvant at week one. Three boosts were subsequently administered by the i.p. route at weeks 2, 3, and 4, consisting of 50 µg of antigen emulsified into 20 µl of Freund's incomplete adjuvant. Mice were sacrificed by cervical dislocation at week 5 and serum was obtained from blood extracted by cardiac puncture. All these procedures were performed in accordance with the AVMA Guidelines (AVMA 2013) and with animal ethics approval number CEID2014028.

Antigenicity of the moss-produced poly-HIV was assessed by means of ELISA assays using total soluble protein extracts. These were obtained by grinding 20 mg of fresh plant material in a mortar, resuspending the material in 200 µL of protein extraction buffer [50 mM Tris (pH 8.0), 40 mM NaCl, 0.1 % Tween 20 and 100 mM PMSF], followed by centrifugation at 12,000 rpm for 10 min. Assay plates were coated overnight at 4 °C with protein extracts diluted (1:8) in a carbonate/bicarbonate buffer (15 mM Na_2CO_3 and 35 mM $NaHCO_3$, pH 9.6). Subsequent steps were alternated with PBST washes. Plates were blocked with 2 % fat-free dry milk for 2 h at room temperature. Primary labeling was performed overnight at 4 °C with the anti-ELDWKA or anti-C4(V3)6 sera (1:250 dilutions). A horseradish peroxidase-conjugated anti-mouse IgG (1:2000 dilutions) was added afterwards and incubated for 1 h. A substrate solution composed of 0.3 mg/l 2-2′-azino-bis (3-ethylbenzthiazoline-6-sulfuric Acid) (ABTS; Sigma-Aldrich, Missouri, USA) was added and the OD at 405 nm was recorded in a Multiskan Ascent micro-plate reader (Thermo Scientific). The poly-HIV content was estimated in terms of the ELDWKA motif by including a standard curve made with known concentrations of the ELDWKA synthetic peptide in a range of 0.02–2.5 ng/µl (Fig. 4b).

Immunogenicity evaluation

Two groups (each $n = 5$) of BALB/c mice (Potter 1985) were established and subjected to subcutaneous administration of moss biomass from line 10 (group poly-HIV) and from an untransformed line (group WT), respectively. An unimmunized mice group was also included as negative control (C group). The mice were subjected to an immunization scheme consisting of one weekly subcutaneous dose during 4 weeks of 10 mg of moss gametophores containing approximately 34 ng of poly-HIV homogenized in 100 µL of PBS right before administration. Mice were sacrificed 1 week after the last immunization and sera obtained from blood were collected by cardiac puncture. Sera were separated by centrifugation at 5,000 rpm for 10 min and stored at −20 °C until further use. An ELISA analysis was conducted to detect specific antibodies in the sera of immunized mice. Plates were coated with 1 µg per well of the ELDWKA synthetic peptide. Incubation steps and signal detection were performed as described above.

Data analysis

Statistical significance ($P < 0.05$) was determined using a one-way ANOVA followed by Tukey's post hoc test performed with the Statistica software. The results are given as mean ± standard deviation.

Results

A gene of 942 bp was designed to encode a poly-HIV protein of approximately 33 kDa as an effort to obtain a multi-epitope antigen capable of eliciting a broad repertoire of anti-HIV antibodies. The construct pCA200P-HIV, which is schematically represented in Fig. 1, was successfully obtained as evidenced by restriction profiles and sequencing results (data not shown). After *Eco*RI/*Hin*dIII digestion, the expression cassette was introduced into *P. patens* by means of protoplast transfection, obtaining, after selection and regeneration, several lines with a hygromycin resistance phenotype. PCR analysis reflected that many of the putative transformed lines carry the transgene since amplicons of approximately 250 bp were detected in lines 6, 7, 8, 9, 10, 15 and 16 (Fig. 2). These seven PCR-positive plants were selected for further analysis. Under all growth conditions tested, we did not observe any morphological difference between these transgenic lines and untransformed wild-type (WT) *P. patens*.

Protein production was analyzed by Western blots. Using either anti-C4(V3)6 or anti-His hyperimmune sera, the expected heterologous poly-HIV protein was detected with an apparent molecular weight (MW) of about 35 kDa, which is in accordance with the theoretical molecular weight (33 kDa). The immunoreactive protein was present in the extracts of all transformed lines, but not detectable in

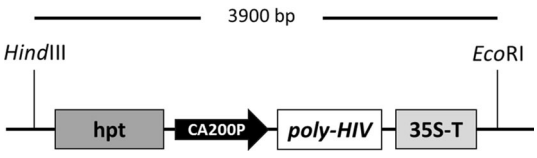

Fig. 1 Graphical representation of the expression cassette used for the insertion and expression of the *poly-HIV* gene in *Physcomitrella patens*. The transgene is under the control of the CA200 promoter. The expression of the *hygromycin phosphotransferase (hpt)* marker gene is mediated by an additional expression cassette allowing for the rescue of transformants

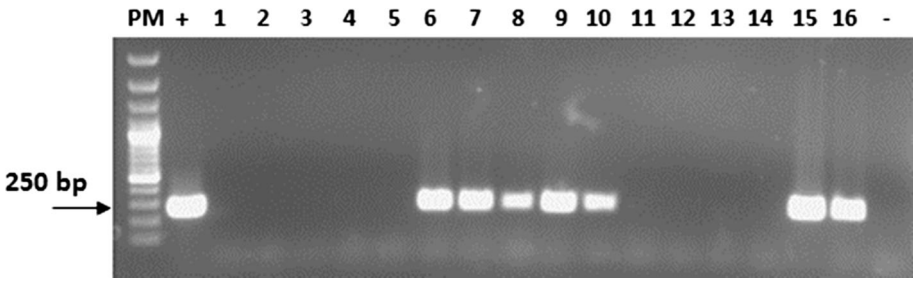

Fig. 2 PCR detection of the *poly-HIV* gene in *Physcomitrella patens*. Genomic DNA samples from either putative transformed or wild-type plants were used to evaluate the presence of the gene by PCR using poly-HIV-specific primers. *PM*, molecular marker; +, positive control; *lanes 1* to *16*, candidate lines; −, wild-type line

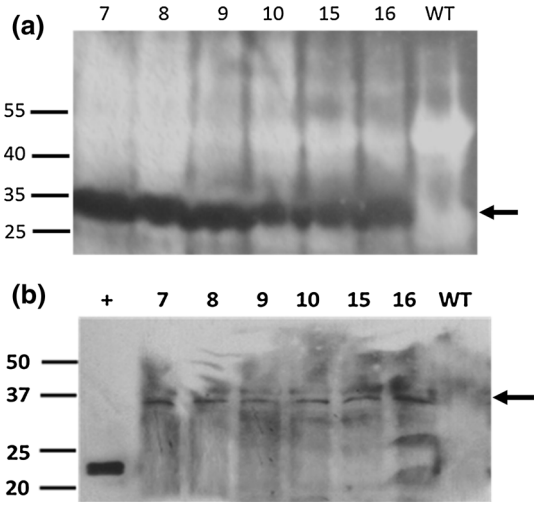

Fig. 3 Production and detection of the immunoreactive poly-HIV protein in *Physcomitrella patens*. The presence of the expected recombinant poly-HIV was determined by Western blot analysis. *Physcomitrella*-derived poly-HIV protein was detected by labeling with either an anti-HIS or an anti- C4(V3)6 hyperimmune serum. **a** Anti-HIS blot. *Lanes* are showing transgenic lines *7*, *8*, *9*, *10*, *15*, *16* and a wild-type sample (WT). Molecular weight in kDa is indicated (PageRuler Prestained Protein Ladder, Thermo Scientific). **b** Anti-C4(V3)6 blot. *Lanes* are showing a positive control for the C4V6 protein (+, 500 ng), transgenic lines *7*, *8*, *9*, *10*, *15*, *16* and a wild-type sample (WT). Molecular weight in kDa is indicated (Dual color, BIORAD). *Arrows* denote protein bands

WT extracts (Fig. 3a, b). Background was always high in Western blots due to the use of total moss extracts.

Antigenicity of the poly-HIV protein was determined by reactivity of an anti-ELDWKA or an anti-C4(V3)6 serum against the poly-HIV extracts. Significant higher OD values ($P < 0.05$) were recorded for the extracts from transformed lines when compared to those of the WT line (Fig. 4a), suggesting a positive recognition of the ELDWKA component present in the poly-HIV protein. A similar finding was made when labeling with the anti-C4(V3)6, reflecting a positive reactivity with the C4 and V3 components (data not shown). The accumulation levels of the ELDWKA motif were calculated based on a standard curve ($R^2 = 0.9903$) (Fig. 4b). Levels of up to 3.7 µg protein per g fresh weight protonema were reached in the moss lines (Fig. 4c).

To get additional insights into the functional properties of the poly-HIV moss-derived protein, an initial immunogenicity test was performed in BALB/c mice. Anti-ELDWKA antibody levels were estimated by ELISA after a subcutaneous immunization scheme with moss tissues from Line 10. Mice immunized with moss-derived poly-HIV elicited significant levels ($P < 0.05$) of anti-ELDWKA antibodies (mean $OD_{405nm} \pm$ standard deviation $= 0.36 \pm 0.059$) as compared to either the WT moss-treated group (mean $OD_{405nm} \pm$ standard deviation $= 0.20 \pm 0.005$) or the C group (Fig. 5).

Discussion

As HIV infection/AIDS continues as an epidemic with high therapy costs, vaccines are one priority to fight this worldwide health problem (Excler et al. 2014). To our knowledge, this study reports for the first time the production of a recombinant vaccine candidate in the moss (*P. patens*) system. The antigenic protein poly-HIV was expressed in transgenic moss lines under the control of a novel moss promoter. Poly-HIV contains several HIV epitopes of relevance for the induction of neutralizing antibodies. We have previously reported the expression of antigenic HIV proteins in tobacco chloroplasts as a model of a low-cost vaccine (Rosales-Mendoza et al. 2014b) and a number of research groups have successfully produced several HIV antigens in other plant systems such as lettuce, tobacco and tomato (Govea-Alonso et al. 2013; Gonzalez-Rabade et al. 2011; Cueno et al. 2010; de Virgilio et al. 2008). The main strength of this focus lies in the fact that oral vaccines are ideal, specifically for developing countries, as these would be low-cost formulations based on freeze-dried biomass. This does not require protein purification and can be easily administered in the form of pills, thus avoiding the need for trained

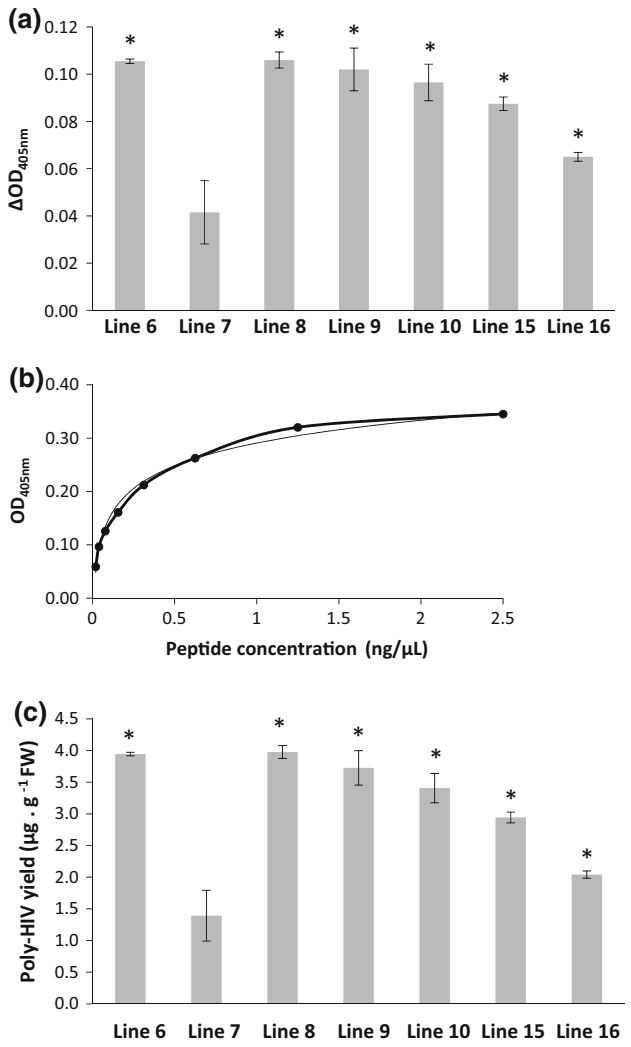

Fig. 4 Antigenicity and yields of the moss-derived poly-HIV protein. **a** An ELISA assay was conducted with total soluble protein extracts from either transformed lines or a WT line as described in materials and methods. Readings from WT samples were subtracted from those of the transgenic lines. Labeling was performed with a hyperimmune serum against the ELDKWA peptide raised in mice. Significant increases in OD values ($P < 0.05$) with respect to the mean OD from the WT line are represented to evaluate poly-HIV antigenicity and marked with *asterisks*. **b** A standard curve using ELDKWA synthetic peptide (0.02–2.5 ng/μl) to estimate the poly-HIV content through the ELDKWA moiety. **c** Poly-HIV yields estimated in the transformed moss clones. *Asterisk* denotes a statistic difference ($P < 0.05$)

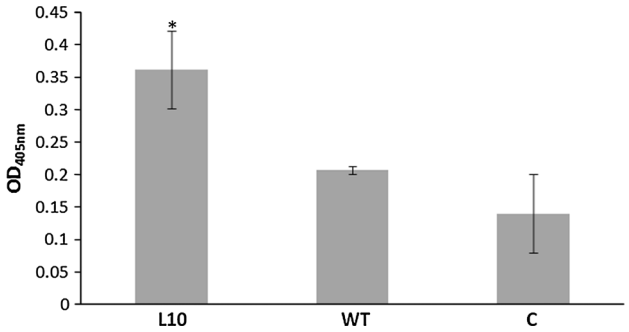

Fig. 5 Immunogenicity evaluation of the moss-derived poly-HIV. BALB/c mice received subcutaneously moss extracts from WT or the poly-HIV expressing Line 10 (L10). *C* represents an unimmunized mice group. Antibody levels were estimated by an ELISA assay where plates were coated with an ELDKWA synthetic peptide. Data represent means of experimental groups ($n = 5$). *Asterisk* denotes a statistic difference ($P < 0.05$) versus the WT group

personnel. Oral vaccines are also better suited to produce mucosal responses that are of key relevance for pathogens whose entry sites are mucosal compartments (Kajikawa et al. 2012).

The moss *Physcomitrella patens* meets the requirements for a safe and flexible biopharmaceutical production platform. Outstanding genetic resources and well-developed molecular tools are available and the predominantly haploid life cycle combined with a high rate of homologous recombination in mitotic cells makes custom-designed, targeted modifications possible (Decker and Reski 2007, 2008; Mueller et al. 2014). The present study focused on moss as a convenient platform with the following advantages: strains can be propagated in vitro under full containment according to GMP conditions and moss biomass has no apparent toxic effects, allowing for immunization with raw moss material. In addition, *P. patens* performs post-translational protein N-glycosylation preferentially of the complex type (Koprivova et al. 2003), identical to that of higher plants, which may have effects on immunogenicity. However, the moss recombination machinery enables precise glycoengineering of the produced vaccine prototypes, so that the effects of glycan variations could be studied in a straightforward manner unlike in any other plant system (reviewed in Rosales-Mendoza et al. 2014a).

Moss was able to successfully synthesize the poly-HIV protein, retaining antigenicity and immunogenicity. In terms of yield, this moss platform allows for the production of up to 3.7 μg g^{-1} fresh weight protonema. Importantly, no toxic effects are apparent in the poly-HIV moss producing lines as their growth rates and morphology were undistinguishable from wild type. Here, we achieved a similar productivity to that observed for other biopharmaceuticals produced at levels ranging from 20 to 300 μg g^{-1} dry weight, such as erythropoietin, human Complement Factor H, human VEGF and tumor antigen-specific antibody IGN314 (Parsons et al. 2012; Weise et al. 2007; Buettner-Mainik et al. 2011; Baur et al. 2005; Schuster et al. 2007). However, all these recombinant proteins had been secreted to the medium and were collected after prolonged cultivation periods.

Immunization of test mice indicated that moss-derived poly-HIV is immunogenic when subcutaneously

administered, eliciting humoral responses against the ELDWKA epitopes included in the multi-epitope design of poly-HIV. In this study, the characterization of poly-HIV has been initiated and will be continued in subsequent studies that will focus on evaluating the breadth and the neutralizing potential of the humoral responses induced by this moss-derived protein.

Our expression approach was based on the secretion signal peptide that directs the protein to the complex processing machinery, allowing for glycosylation and complex folding in the ER and Golgi apparatus. Considering that poly-HIV has an N-glycosylation site, the production of distinct glycoforms and the analyses of their impact on immunogenicity are envisioned for subsequent studies. A secretion of proteins to the medium offers great advantages for downstream processing as the absence of most contaminating proteins and other metabolites in the supernatant allows easier protein purification. However, an oral vaccine would be desirable and it would have greater advantages over subcutaneous or intramuscular vaccines. This is an unexplored area and thus forthcoming studies have to determine whether *P. patens* is an appropriate edible system or not (Rosales-Mendoza et al. 2014a).

Previous reports on the production of a chloroplast-derived p24-Nef fusion protein from HIV have revealed that the plant-made antigen elicited a strong antigen-specific serum IgG response when subcutaneously administered to mice (Gonzalez-Rabade et al. 2011). Also, other plant-produced recombinant proteins carrying Env epitopes have been tested, proven to be immunogenic in animals and were capable of eliciting humoral and cellular responses (Rosales-Mendoza et al. 2012). These results indicate that antigens produced in plants are promising candidates as components of HIV vaccines.

Taken together, we explored for the first time the recombinant production of an immunogenic protein in moss and suggest that *P. patens* is a suitable platform for the production of vaccines.

Author contribution statement S.R.-M., E.L.D. and R.R. designed and supervised the study. E.L.D. designed and constructed the poly-HIV expression vector. S.R.-M. and J.P. generated and L.O.-E. and J.P. characterized the moss clones. E.M.-E. determined protein expression levels. A.R.-M. evaluated immunogenicity. L.M.-F. collaborated on antigen design. S.R.-M. and R.R. wrote the manuscript with the help of L.O.-E. and E.L.D. All authors discussed the results, read and approved the final version of the manuscript.

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Conflict of interest The authors declare no conflict of interests.

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