

Towards the development of an oral vaccine against porcine cysticercosis: expression of the protective HP6/TSOL18 antigen in transgenic carrot cells

Elizabeth Monreal-Escalante¹ · Dania O. Govea-Alonso¹ · Marisela Hernández² · Jacquelynne Cervantes² · Jorge A. Salazar-González¹ · Andrea Romero-Maldonado¹ · Gabriela Rosas³ · Teresa Garate⁴ · Gladis Fragoso² · Edda Sciuotto² · Sergio Rosales-Mendoza¹

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Abstract

Main conclusion The *Taenia solium* HP6/TSOL18 antigen was produced in carrot cells, yielding an immunogenic protein that induced significant protection in an experimental murine model against *T. crassiceps* cysticercosis when orally administered. This result supports the potential of HP6/TSOL18-carrot as a low-cost anti-cysticercosis vaccine candidate.

Cysticercosis is a zoonosis caused by *Taenia solium* that can be prevented by interrupting the parasite life cycle through pig vaccination. Several injectable vaccine candidates have been reported, but the logistic difficulties and costs for its application limited its use in nationwide control programs. Oral plant-based vaccines can deal with this limitation, because of their easy administration and low cost. A stable expression of the HP6/TSOL18 anti-*T. solium* cysticercosis protective antigen in carrot calli

transformed with an optimized transgene is herein reported. An antigen accumulation up to 14 $\mu\text{g g}^{-1}$ of dry-weight biomass was achieved in the generated carrot lines. Mouse immunization with one of the transformed calli induced both specific IgG and IgA anti-HP6/TSOL18 antibodies. A statistically significant reduction in the expected number of *T. crassiceps* cysticerci was observed in mice orally immunized with carrot-made HP6/TSOL18, in a similar extent to that obtained by subcutaneous immunization with recombinant HP6/TSOL18 protein. In this study, a new oral plant-made version of the HP6/TSOL18 anti-cysticercosis vaccine is reported. The vaccine candidate should be further tested against porcine cysticercosis.

Keywords Cysticercosis · Delivery system · HP6/TSOL18 · Oral vaccine · Plant-based vaccine · *Taenia* · Vaccination

Abbreviations

ERT	Enzyme replacement therapy
GST-HP6/TSOL18	Glutathione <i>S</i> -transferase-HP6/TSOL18 fusion protein
NC	Neurocysticercosis
CNS	Central nervous system

Introduction

Cysticercosis is a parasitic disease, caused by the establishment of the metacystode stage of *Taenia solium* in muscular or nervous tissues. It affects humans and pigs in developing countries where conditions that favor the parasite life cycle prevail (De Aluja 2008). The most severe form of the disease occurs when larvae (cysticerci) lodge in

✉ Edda Sciuotto
edda@unam.mx

✉ Sergio Rosales-Mendoza
rosales.s@fcq.uaslp.mx

¹ Laboratorio de Biofarmacéuticos Recombinantes, Facultad de Ciencias Químicas, Universidad Autónoma de San Luis Potosí, Av. Dr. Manuel Nava 6, 78210 San Luis Potosí, Mexico

² Dpto. Inmunología. Instituto de Investigaciones Biomédicas, Universidad Nacional Autónoma de México, Circuito Escolar, Ciudad Universitaria, 04510 Mexico, D.F., Mexico

³ Facultad de Medicina, Universidad Autónoma del Estado de Morelos, Cuernavaca, Mexico

⁴ Dpto. de Parasitología, Centro Nacional de Microbiología, Instituto de Salud Carlos III, Majadahonda, 28220 Madrid, Spain

the human central nervous system (CNS), causing neurocysticercosis (NC) (Garcia et al. 2005).

With regard to vaccination, a number of highly protective, field-trial tested vaccine antigens have been reported, all of them administered by injection (Huerta et al. 2001; Morales et al. 2008; Assana et al. 2010; Jayashi et al. 2012). Research and development, as well as the approval process, demand high investments that are subsequently recovered with higher-cost vaccines. In addition, production and delivery costs also have a high impact on vaccination campaigns (Gandhi et al. 2013). All these factors limit vaccine coverage in developing countries.

A control program comprising a widespread application of the S3Pvac-phage anti-cysticercosis vaccine along with a health education program conducted for 3 years in a region of southern Mexico proved to reduce cysticercosis transmission effectively (De Aluja et al. 2014). The results of this program made clear the obstacles for the widespread application of an injectable vaccine (Larralde and Sciuotto 2006). A needle-free oral vaccine could cope these difficulties, representing thus a more realistic immunization approach, avoiding costly logistics problems and suitable for nationwide vaccination programs (Hernández et al. 2014). In addition, oral immunization-elicited mucosal immunity offers a first barrier against infective agents entering the body by oral ingestion. The concomitant systemic immunity induced by oral vaccination could then damage those oncospheres that eluded mucosal immunity (Dabral et al. 2014). The viability of this technology is supported by the fact that a number of companies are pursuing the development of plant-based vaccines, with some candidates close to marketing (Yusibov et al. 2011).

Conventional vaccine production platforms require expensive reactors and extensive purification steps during production. On the other hand, plant-based vaccine production platforms offer a significant reduction in production costs that do not require sophisticated bioreactors or purification process (Batson 1998; Berman and Giffin 2004). It has been estimated that the cost of raw materials for plant systems is 10 times lower than that of the *E. coli*-based systems (Streatfield and Howard 2003; Gecchele et al. 2015). In conjunction with government investment, plant-based vaccines may play a key role on achieving proper vaccination coverage (Daniell 2006).

In this context, carrot is a convenient plant species, since undifferentiated carrot cells can be grown in bioreactors with low-cost culture media, yielding safe biomass that can be easily stabilized by a freeze-drying process, for instance (Streatfield and Howard 2003). Several antigens have been produced in carrot, including antigenic proteins from measles virus, the B subunit of the *Escherichia coli* heat-labile enterotoxin (LTB), and the F1 and V antigens from *Yersinia pestis*. Expression levels were sufficient to induce

immune responses in mice (Rosales-Mendoza et al. 2007, 2011) or even immunoprotection against *E. coli* heat-labile enterotoxin (Rosales-Mendoza et al. 2008). Currently, Protalix BioTherapeutics (Carmiel, Israel) is marketing a plant-derived product named ELELYSO, a recombinant hydrolytic lysosomal glucocerebrosidase-specific enzyme for long-term enzyme replacement therapy (ERT) for adults with confirmed diagnosis of type-1 Gaucher's disease. This case exemplifies the potential of the plant-made biopharmaceuticals produced in carrot cell suspensions (Aviezer et al. 2009).

A plant-based anti-cysticercosis vaccine was developed in our research group, using transgenic embryogenic papaya cells. S3Pvac-papaya induced high protection levels against *T. crassiceps* and *T. pisiformis* cysticercosis when parenterally or orally administered (Hernández et al. 2007; Betancourt et al. 2012). This vaccine promoted an exacerbated specific cellular immunity that could mediate the observed protection. On the other hand, the HP6/TSOL18 vaccine candidate antigen, initially identified in *T. ovis* (131 aa) and *T. saginata* (131 aa) (Benitez et al. 1996; Harrison et al. 1996), and subsequently found in *T. solium* (130 aa) (Gauci et al. 1998), induced protection through an exacerbated humoral immunity. (Flisser et al. 2004). Its effective expression in a delivery system adequate for its oral administration will offer two feasible oral vaccine candidates that may be used in complementary schedules to favor the two main branches of immune response. Thus, the potential of the carrot-made HP6/TSOL18 antigen in terms of expression levels, immunogenicity, and protection efficiency is herein investigated.

Materials and methods

Gene design and vector construction

The sequence of the *hp6/tsol18* gene, based on the previously described *T. solium* genome (Parkhouse et al. 2008), was optimized for codon usage in carrot cells, and flanking restriction sites *Sma*I and *Sac*I were inserted to facilitate cloning. The *hp6/tsol18* gene was produced by Genscript (Piscataway, NJ, USA). Then, *hp6/tsol18* was cloned into the pBI121 binary vector downstream of the CaMV 35S promoter following conventional cloning procedures, to yield the expression vector named pBin-HP6/TSOL18. After transforming chemically competent *E. coli* cells, a positive clone was selected by restriction analysis and used for plasmid isolation. After confirming sequence integrity by standard sequencing, the pBin-HP6/TSOL18 construct was mobilized into the *Agrobacterium tumefaciens* GV3101 strain by electroporation, and this recombinant strain was used for explant carrot transformation.

Genetic transformation of carrot stems

Carrot transformation was carried out following a previously described method (Rosales-Mendoza et al. 2007). Briefly, carrot (*Daucus carota* L.) seeds (Eden, Lower Beechmont, QLD, Australia) were surface-sterilized with 50 % commercial Clorox bleach for 15 min and washed five times in sterile distilled water (5 min per rinse). Seeds were then treated with HCl 0.01 M in sterilized distilled water for 15 min, washed five times with sterile distilled water, and then germinated in 15 × 100 mm glass jars containing Murashige and Skoog (MS) (1962) medium. Cultures were maintained under a 16-h photoperiod (100 μmol m⁻² s⁻¹) at 25 °C for 4–5 weeks. Stems from carrot seedlings were cut into 7–10-mm segments on wet sterile filter paper. Explants were incubated in an overnight-grown culture of *Agrobacterium* (OD_{600nm} = 0.5) for 15 min, transferred onto the co-cultivation medium [MS basal medium supplemented with 1.33 μM 6-benzyladenine (BA) and 4.52 μM 2,4-dichlorophenoxyacetic acid (2,4-D)], and co-cultivated in the dark for 48 h. Explants were thoroughly washed with sterilized-deionized water and cultured on selection medium (MS basal medium supplemented with 1.33 μM BA and 4.52 μM 2,4-D along with 500 mg/l cefotaxime and 50 mg/l kanamycin). All cultures were grown under a 16-h photoperiod (100 μmol m⁻² s⁻¹) at 25 °C. Explants were subcultured once every 2 weeks onto fresh selection medium. Kanamycin-resistant calli were propagated in selective media over a period of 4 months to obtain enough amounts for further analysis. Biomass from candidate lines was collected, freeze-dried overnight, and milled in an analytical grinder.

Transgene analysis

To confirm the presence of the transgene in candidate carrot lines, a PCR assay was performed. Total genomic DNA was isolated from putatively transformed calli lines and wild-type calli, according to Dellaporta et al. (1983). A 25-μl reaction mixture containing 100 ng of DNA, 1.5 mM magnesium chloride, 2.5 U Taq DNA polymerase, 1 mM dNTPs, and 1 μM primers (Sigma, <http://www.sigmaldrich.com>) was prepared. Two primer sets were used to detect the selectable marker *nptII* gene (forward, 5'TATTCGGCTATGACTGGGCA3'; reverse, 5'GCCAACGCTATGTCCTGAT3') or the TEV-UTR sequence (forward 5'TCTAGAAACACAACATATACAAAACAAACGA3'; reverse 5'CCCGGGCTATCGTTCGTAATGGTGAAA3'). Amplification protocol comprised an initial denaturation at 95 °C for 3 min and 35 cycles including incubations at 95 °C for 30 s, 55 °C for 60 s, and 72 °C for 90 s, followed by a final extension at

72 °C for 5 min. PCR products were analyzed by electrophoresis in 1 % agarose gels.

Determination of transgene copy number by real-time PCR

To determine the number of transgene copies in the transformed carrot lines, a real-time PCR (q-PCR) protocol was performed following the method reported by Weng et al. (2004) with some modifications. Two primer sets were used to amplify the gene *nptII*, which is present in the T-DNA region (sense 5'ATGCCTGCTTGCCGAATA3'; antisense 5'AGCGTGAGGAGTCTTGTATTTC3'), and the *DcPRP1* gene, which codes for a wound-inducible, proline-rich cell wall protein, serving as single-copy gene control (sense 5'CAACCACCGAAGACTGAGAAG3'; antisense 5'GGAGGATTAGGCTTGTGTGTAG3'). Primers were designed using the IDT PrimerQuest tool (www.idtdna.com). PCR was carried out in 48-well reaction plates (Applied Biosystems, Waltham, MA, USA) using the SUPER SYBR Mix (Applied Biosystems) with primers at a final concentration of 200 nM and a 10-μl final volume. PCR reactions were performed in a StepOne™ Real-Time PCR system (Applied Biosystems) under the following cycling conditions: 95 °C for 10 min (initial denaturation), 40 cycles at 95 °C for 15 s for denaturation, and 60 °C for 60 s for annealing and extension. PCR specificity was confirmed by melting-curve analysis to ensure the absence of non-specific amplicons and primer dimers. To construct standard curves, target or endogenous genes were amplified by duplicate from transgenic line 3, and 7 genomic DNA concentrations randomly chosen, ranging from 1.56 to 100 ng/μl (set by twofold serial dilutions). To determine the number of transgene copies, PCR reactions were performed by triplicate with 15 ng of DNA from transgenic lines 3, 4, and 5, or the WT carrot line. Ct values for the transgene and endogenous genes were determined under the same auto baseline and threshold. Results were analyzed using the StepOne™ Software v2.1.

Transgene copy number was calculated using the following equation:

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

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 Ct,X and Ct,R are the Ct values of the target and the reference gene, 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 Ct,X, Ct,R, SX, SR, IX, and IR values obtained from the standard curve.

Protein analysis

HP6/TSOL18 expression was assessed by a Western blot assay in the candidate carrot calli lines. Protein extracts were obtained by resuspending 50 mg of freeze-dried callus tissue in 50 μ l of 1X reducing loading buffer. *E. coli*-produced GST-HP6/TSOL18 recombinant protein was used as positive control. Samples were denatured by boiling for 5 min at 95 °C, debris were eliminated by centrifugation at 16,000g for 15 min at 4 °C, and SDS-PAGE was performed in 4–12 % acrylamide gels under denaturing conditions. The gel was blotted onto BioTrace PVDF membranes (Pall Corporation, <http://www.pall.com>). After blocking with PBS-Tween 0.1 % plus 5 % fat-free milk (Carnation, Nestle, <http://www.nestle.com>), blots were incubated overnight with a polyclonal rabbit antibody against the recombinant protein GST-HP6/TSOL18 (1:800 diluted in saline). Thereafter, blots were incubated with a goat anti-rabbit antibody horseradish peroxidase-conjugate at a 1:2000 dilution (Sigma) for 2 h at room temperature to visualize the specific antibody bound. Antibody binding was detected with the SuperSignal West Dura Extended Duration Substrate solution, following the manufacturer's instructions (Thermo Scientific, [http://www.thermo scientific.com](http://www.thermoscientific.com)) and by means of an X-ray film, following standard procedures.

To estimate the amount of protein produced in carrot cell lines, 50 mg of lyophilized callus tissue was grinded and suspended in 500 μ l of protein extraction buffer (50 mM Tris pH = 8, 40 mM NaCl, 0.1 % Tween 20, 0.05 % PMSF). Samples were centrifuged at 16,000g for 15 min at 4 °C. ELISA assay plates were then coated overnight at 4 °C with protein extracts diluted 1:2 in carbonate buffer (0.2 M, pH = 9.6). Plates were washed three times with 0.1 % PBST and blocked with 5 % fat-free dry milk in PBS for 2 h at room temperature. After washing, polyclonal rabbit anti-GST-HP6/TSOL18 diluted 1:800 in PBS was added and plates were incubated overnight at 4 °C. A goat anti-rabbit antibody horseradish peroxidase-conjugated diluted 1:2000 (Sigma) was added and incubated for 2 h at 25 °C. After washing, a substrate solution of 0.3 mg/ml 2-20-azino-bis-3 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) acid (ABTS; Sigma) and 0.1 mM H₂O₂ was added for 30 min at 25 °C. Optical density (OD) was read in an iMark™ microplate reader (Bio-Rad, Hercules, CA, USA) at 410 nm. GST-HP6/TSOL18 recombinant protein was used to make a standard curve to determine HP6/TSOL18 level expression in each carrot cell line and was expressed as μ g of HP6/TSOL18 per gram of dried biomass (μ g g⁻¹ DW).

Mouse immunization

All experiments herein reported were conducted in accordance with the principles set forth in the Guide for the Use of Laboratory Animals, Institute of Laboratory Animal Resources, National Research Council, Washington, DC, USA, 1996. All experimental protocols were approved by the Animal Care Committee of the University and followed the Guide for Care and Use of Experimental Animals.

Groups of ten 8–11-week-old female BALB/cAnN mice were subcutaneously immunized at the back of the neck three times at days 1, 7, and 14 with 10 mg of freeze-dried calli resuspended in 100 μ l of PBS. To improve the antibody response, 2 weeks after the last s.c. immunization, mice received an oral boost of 50 mg of freeze-dried transgenic calli material (carrot line 5) (containing approximately 700 ng of recombinant HP6/TSOL18) or the wild-type line suspended in 300 μ l of PBS. Two weeks later, mice were sacrificed, and serum samples as well as intestinal washes were collected for antibody detection. The latter were obtained by resecting the intestines and flushing through them 1 ml of cold PBS supplemented with 1 mM PMSF. The material was collected and samples were centrifuged at 16 000g at 4 °C for 10 min. Supernatants were stored at –70 °C.

Anti-HP6/TSOL18 antibody detection

Antibody levels were assessed by ELISA using microtiter plates (Costar, Corning Incorporated, Corning, NY, USA) coated with 300 ng of recombinant GST-HP6/TSOL18 in 100 μ l of carbonate buffer 0.2 M, pH 9.6, and incubated overnight at 4 °C. Plates were washed with 0.05 % PBST and blocked for 2 h at room temperature with 5 % fat-free dry milk dissolved in PBS. To determine serum antibody levels, plates were incubated with 1:20 diluted sera and incubated overnight at 4 °C. Either horseradish peroxidase-conjugated goat anti-mouse IgG or horseradish peroxidase-conjugated goat anti-mouse IgA (1:2000 dilution; Sigma) was added and incubated for additional 2 h at room temperature. After washing with 0.05 % PBST, 100 μ l of a substrate solution of 0.3 mg l⁻¹ ABTS (Sigma) and 0.1 mM H₂O₂ was added and incubated for additional 20–30 min at room temperature. OD at 410 nm was recorded in an iMark™ microplate reader (Bio-Rad).

HP6/TSOL18 histological immunolocalization

Taenia crassiceps cysticerci recovered from the peritoneal cavity of BALB/cAnN female mice were treated using the procedure previously described by Rosas et al. (1998) to dissociate non-specifically bound host proteins. Briefly, vesicular fluid was removed, the cyst was thoroughly washed with ice-cold PBS and incubated with 50 mM

glycine–HCl, pH 2.5; 0.1 % Triton X-100; 0.15 mM NaCl for 30 s; pH was restored by adding Tris–HCl, pH 9, and cyst was fixed for 72 h in Zamboni solution. Afterwards, the specimens were included in paraffin and 6 μ m sections were cut. Slides were placed on poly-L-lysine-treated microslides (Sigma). Peroxidase activity was blocked by treatment with 3 % H₂O₂ in PBS for 10 min at room temperature. After washing with PBS, the slides were blocked with 5 % BSA in PBS plus 0.1 % Triton X-100 (pH 7.4) for 1 h at 37 °C. Solutions were removed and the slides were incubated overnight at 4 °C either with rabbit pre-immune sera or with rabbit hyper-immune anti-HP6/TSOL18 sera. Those sera were employed as primary antibody source at a dilution of 1:1000 in 1 % BSA in PBS plus 0.1 % Triton X-100 (PBS/A-T). After washing three times in PBS/A-T, 5 min each, slides were covered with a biotinylated goat anti-rabbit IgG (ImmunO Universal Kit, MP Biomedicals, Eschwege, Germany) for 30 min at 37 °C, rinsed with PBS/A-T, and treated with streptavidin–peroxidase conjugate (HRP conjugated, Universal Kit, MP Biomedicals) for 30 min at 37 °C. Peroxidase activity was visualized by incubating the samples with 3′3-diaminobenzidine (DAB-Plus Kit, Zymed, South San Francisco, CA, USA). The slides were counterstained with Mayer’s hematoxylin, dehydrated, cleared, mounted, and observed through an optical microscope (Nikon, Tokyo, Japan) using the MetaMorph Imaging System v.4.5 software.

Mouse immunization for cysticercosis protection studies

The immunogenicity of the carrot-derived HP6/TSOL18 (line 5) was evaluated in female BALB/cAnN mice (6–8 weeks-old), cared for in accordance with federal regulations for animal experiments (NOM-062-ZOO-1999, Ministry of Agriculture, Mexico). The immunization protocol was approved by the Institutional Animal Care Committee.

Mice were divided into three different groups ($n = 7$), each receiving one of the following treatments: total soluble protein extracted from 10 mg of freeze-dried HP6/TSOL18 transgenic line 5 administered orally by gavage, or as a

recombinant GST-HP6/TSOL18 expressed in *E. coli* by s.c. immunization. Control mice were orally immunized with soluble extract derived from 10 mg of a non-transformed carrot line (WT group). Subsequently, mice were boosted at day 10 and challenged 2 weeks after the last immunization.

Parasite and infections

Parasites for challenge infection were obtained from 2 to 3-month-old intraperitoneally infected mice as previously described (Toledo et al. 2001). Two weeks after the last immunization, mice were intraperitoneally infected with 20 small (2 mm in diameter), non-budding *T. crassiceps* cysticerci in 0.9 ml of PBS. Mice were sacrificed 30 days after infection, and cysts were harvested from peritoneal cavity and counted as previously reported (Fragoso et al. 2011). The effect of vaccination was evaluated by the mean number of cysticerci recovered from each mouse.

Statistical analysis

Data were analyzed by one-way ANOVA analysis using the STATISTICA 2.7 software, and Student’s *t* tests for independent samples. Differences were regarded as significant for $P < 0.05$.

Results

Expression of HP6/TSOL18 antigen in transgenic carrot cells

In this study, the pBI121 vector was selected to drive the transfer and expression of a synthetic *hp6/tsol18* gene. The strategy, based on the use of restriction sites with *Sma*I and *Sac*I, allowed the successful cloning of the gene into the pBI121 vector, yielding the pBin-HP6/TSOL18 construct, as confirmed by sequencing. A positive clone was propagated and used for *Agrobacterium*-mediated transformation. This vector is schematically represented in Fig. 1.

After transformation procedure by *Agrobacterium* infection, multiple independent kanamycin-resistant (Kan^R) carrot calli were generated 1 month after co-

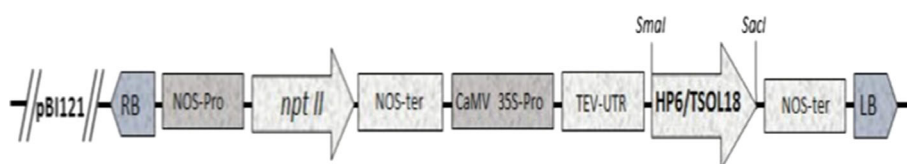


Fig. 1 Map of the binary vector pBin-HP6/TSOL18 constructed to express the *hp6/tsol18* gene in carrot calli. The gene of interest *hp6/tsol18* is under control of the CaMV 35S promoter, along with the

5′UTR from tobacco etch virus (TEV). In addition, an *nptII* expression cassette is included to allow for kanamycin-based selection of transformed lines

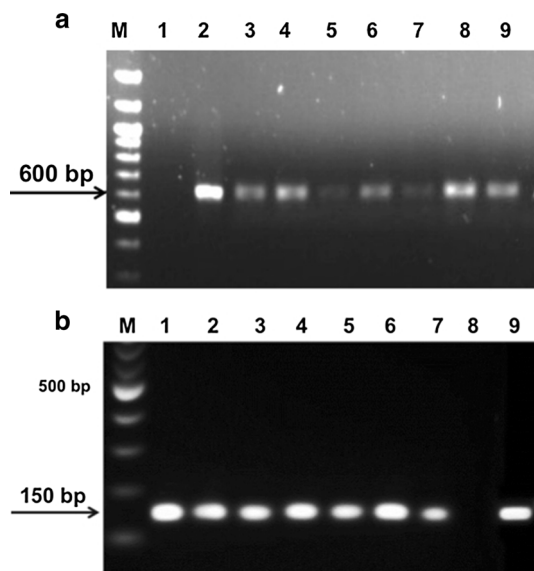


Fig. 2 Detection of *hp6/tsol18* expression cassette in transformed carrot lines by PCR. **a** Detection of the *nptII* gene. PCR analysis was conducted using genomic DNA extracted from transgenic or wild-type calli and *nptII*-specific primers. Lanes *M* 100-bp ladder (New England Biolabs); 1 WT wild-type callus; 2 positive control (plasmid pBin-HP6/TSOL18); 3–9 transgenic lines 1–7, respectively. **b** Detection of *TEV* UTR. PCR analysis was conducted using genomic DNA extracted from transgenic or wild-type calli and *TEV*-UTR-specific primers. Lanes: *M* 100-bp ladder; 1–7 transgenic lines 1–7 respectively; 8 WT wild-type callus; 9 positive control (plasmid pBin-HP6/TSOL18)

cultivation. The *nptII* and *TEV*-UTR regions present in the T-DNA were successfully amplified in all lines, as reflected by the presence of 600-bp amplicons for the *nptII* transgene (Fig. 2a), and 150-bp amplicon for the *TEV*-UTR region (Fig. 2b), thus confirming the presence of the heterologous DNA in the carrot lines. No PCR product was amplified in reactions with DNA from wild-type calli.

Lines 3, 4, and 5 were selected to determine the number of transgene copies by q-PCR. To assess the number of transgene copies in the carrot line genome, standardized curves were obtained from the q-PCR analysis for the *nptII* and *DcPRP1* genes in serial dilutions of genomic DNA samples. Slopes (*S*) and intercepts (*I*) derived from standard curves for both transgene and endogenous genes were calculated to determine the copy number. *S* and *I* values for the *nptII* primers curve were -3.578 and 30.2 , respectively, with a 90.92% primer efficiency. For the *DcPRP1* primers curve, *S* and *I* values were -3.58 and 31.9 , respectively, with a 91.53% primer efficiency. Correlation coefficients of the standard curves for both genes showed acceptable values (0.9998 and 0.9982 , respectively). Table 1 shows Ct mean values for the transgenic and WT carrot lines. Based on these data, it can be concluded that a single insertion of the transgene occurred in the selected transgenic lines.

Table 1 Results of qPCR analysis to estimate the number of T-DNA insertions in carrot lines

Sample	Ct mean <i>DcPRP1</i>	Ct mean <i>T-DNA</i>	Copy number
Line 3	25.45	23.96	1
Line 4	26.75	25.20	1
Line 5	25.30	23.74	1
WT	29.97	Undetermined	–

The *DcPRP1* gene was used as a single-copy reference gene

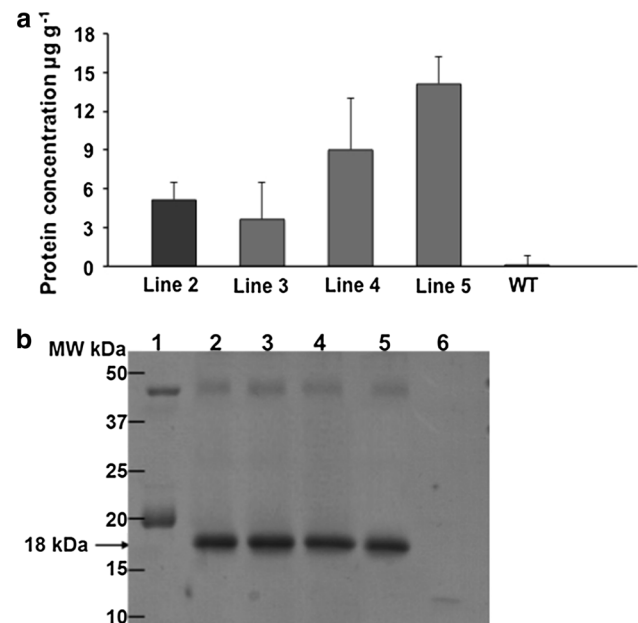
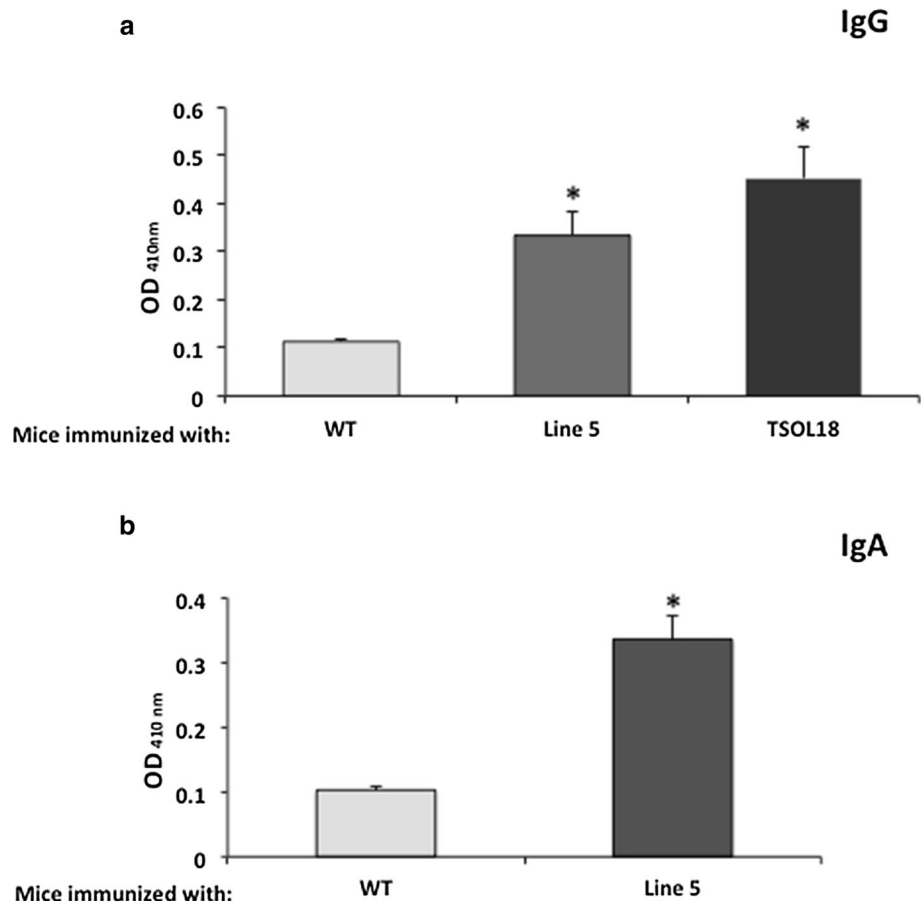


Fig. 3 **a** Accumulation levels of the HP6/TSOL18 protein in carrot calli lines detected by ELISA. Carrot lines 2–5 showed HP6/TSOL18 expression. **b** Immunodetection of the HP6/TSOL18 antigen in carrot total protein extracts. Western blot was conducted using a rabbit polyclonal anti-GST-HP6/TSOL18 antibody to assess the presence of the HP6/TSOL18 protein in total protein calli extracts. Lanes 1 purified recombinant GST-HP6/TSOL18 (positive control, 500 ng); lanes 2–5 protein extracts from transgenic carrots lines 2, 3, 4, 5; lane 6 protein extract from wild-type carrot callus

To assess the ability of transgenic carrot cell lines to synthesize the recombinant HP6/TSOL18 protein, an initial ELISA screening was performed. Carrot lines 2–5 showed higher HP6/TSOL18 expression (Fig. 3a), and therefore, these four lines were selected to detect the recombinant protein by Western blot. Rabbit anti-GST-HP6/TSOL18 sera detected the presence of the expected 18-kDa protein in samples from transgenic carrot lines (Fig. 3b, lines 2–5). In addition, extracts from transgenic lines showed a band of higher molecular weight, approximately 40-kDa. The positive control showed a 40-kDa protein, in agreement with the theoretical molecular weight of the GST-HP6/

Fig. 4 Levels of anti-HP6/TSOL18 IgG antibodies detected by ELISA in sera (a), IgA antibodies detected in intestinal washes (b) from mice immunized either with recombinant GST-HP6/TSOL18, line 5 carrot callus, or wild-type carrot callus (WT). Asterisk denotes statistical difference with respect to the WT-immunized group ($P < 0.05$)



TSOL18 fusion protein, while an unexpected 20-kDa band, which might correspond to a degradation product, was also observed.

Line 5 was selected for further analysis. HP6/TSOL18 expression levels in this line were confirmed by ELISA. OD values for the line 5, significantly higher than those of the WT carrot line, were used to estimate accumulation levels according to the standard curve obtained with recombinant GST-HP6/TSOL18. Expression levels for line 5 were estimated in 14 $\mu\text{g g}^{-1}$ dry-weight of carrot calli.

Carrot-derived-HP6/TSOL18 is highly immunogenic in mice

To analyze the immunogenic potential of the carrot-based material expressing the HP6/TSOL18 protein, either calli from the transgenic line 5 or WT calli were used to immunize groups of BALB/cAnN mice. The levels of local and systemic antibodies induced by immunization with the transgenic callus line 5 were estimated by ELISA and compared to those induced by immunization with wild-type callus. This is an acceptable and widely used method for this purpose, since the statistical analysis validates the difference between treatments. According to the statistical analysis of

the OD values, significantly higher levels of anti-HP6/TSOL18 IgG serum antibodies were detected in mice immunized with freeze-dried tissue from the line 5 or pure recombinant GST-HP6/TSOL18 ($P < 0.05$; Fig. 4a). Moreover, an increase in anti-HP6/TSOL18 mucosal IgA antibody levels was detected in the intestines of mice orally boosted with the transformed carrot line 5 ($P < 0.05$; Fig. 4b).

Anti-TSOL18 antibodies recognized several structures of *T. crassiceps cysticerci*

As shown in Fig. 5, anti-HP6/TSOL18 antibodies specifically recognized several structures of *T. crassiceps cysticerci*: Tegument (T), parenchyma (P), distal cytoplasm region (DC), and the perinuclear cytoplasm region (PC). No reaction was detected using pre-immune sera.

Protective effect of oral immunization with the HP6/TSOL18-carrot line 5 against murine cysticercosis

The effect in mice of oral immunization with total extract of freeze-dried calli of HP6/TSOL18-expressing carrot line 5 or wild-type carrot alone is shown in Table 2. Orally administered HP6/TSOL18-carrot significantly reduced the

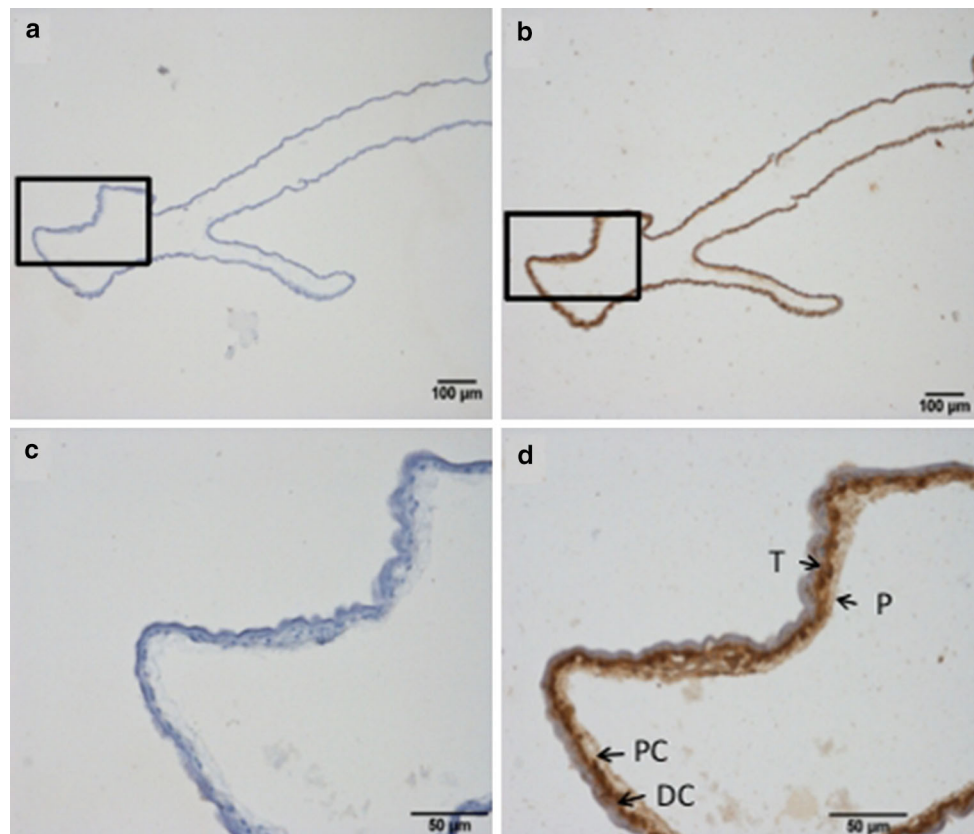


Fig. 5 Immunohistochemical staining of *Taenia crassiceps* (ORF) metacystodes. Sections were incubated with rabbit sera before and after immunization with the recombinant HP6/TSOL18 protein. Bound antibodies were detected using biotinylated goat anti-rabbit IgG plus streptavidin-peroxidase conjugate, and counterstained with

hematoxylin. **a** and **c** Controls incubated with rabbit pre-immune sera. **b** and **d** Cysticerci structures recognized by the rabbit anti-recombinant GST-HP6/TSOL18 sera. *T* tegument; *P* parenchyma; *DC* distal cytoplasm region; *PC* perinuclear cytoplasm region

Table 2 Protection against murine *Taenia crassiceps* cysticercosis

Mice immunized with:	Number of cysticerci per mouse	Mean \pm SD	<i>P</i>
WT carrot (control)*	106, 41, 0, 257, 134, 108 [†]	107.6 \pm 98.8	
Line 5 carrot-HP6/TSOL18*	97, 23, 4, 0, 25, 0, 2	21.6 \pm 34.9	0.036
Recombinant GST-HP6/TSOL18**	3, 0, 3, 6, 0, 119	21.8 \pm 47.6	0.062

[†] Total number of cysticerci harvested from the peritoneal cavity of each mouse. The *P* value obtained by comparison between control and each of the immunized group of mice using the unpaired *t* test

Mice were *orally or **subcutaneously immunized

expected parasite load at a similar level to that of GST-HP6/TSOL18 recombinantly expressed in a bacterial system and s.c. administered. When orally administered, non-transgenic carrot calli alone failed to reduce the expected parasite load.

Discussion

In this study, carrot was selected as a convenient platform to explore the development of an affordable oral vaccine based on the *T. solium* HP6/TSOL18 antigen. It is

remarkable that after transformation (as confirmed by conventional PCR) with a single T-DNA insertion (as confirmed by qPCR), carrot cells synthesized and accumulated the HP6/TSOL18 antigen, which was detected by both ELISA and Western blot analysis. Even though all assessed lines carry a single T-DNA copy, they showed different antigen yields, which may be due to differential transgene insertion sites into the carrot genome (Kim et al. 2007). The unexpected 40-kDa band observed in Western blot may correspond to HP6/TSOL18 associated to endogenous carrot proteins or a dimeric form of the antigen, since it was not observed in the wild-type cell line.

Expression levels of up to 14 $\mu\text{g TSOL18 g}^{-1}$ dry-weight carrot biomass were achieved (line 5), which are in the expected range according to previous experiences with carrot-produced antigens. For instance, the B subunit of *E. coli* heat-labile enterotoxin (LTB) accumulated at 23 $\mu\text{g g}^{-1}$ dry-weight levels (Marquet-Blouin et al. 2003; Rosales-Mendoza et al. 2008).

Early efforts to conduct anti-cysticercosis vaccination programs were hampered by difficulties associated with antigen production and the limited effectiveness of crude parasite extracts as vaccines, as well as prohibitive production costs. For this reason, innovations to override such limitations are much-needed (Molinari et al. 1993, 1997; Wang et al. 2003; Wu et al. 2005; Lightowlers 2006; Cai et al. 2008; Sciutto et al. 2008). The introduction of recombinant vaccines and robust production systems yielded promising vaccine candidates that have been evaluated in field trials (Huerta et al. 2001; Sciutto et al. 2007; Morales et al. 2008; Assana et al. 2010). Carrot cells can be propagated and cultured at industrial scale in bioreactors under well-established GMP-compliant procedures, as exemplified by the case of Protalix, a carrot-made glucocerebrosidase used to treat Gaucher's disease (Aviezer et al. 2009); this successful case points out the potential of this platform for the production and delivery of biopharmaceuticals, preserving their biological activity. In our case, the immunogenic activity of plant-made HP6/TSOL18 was evaluated in BALB/cAnN mice. A successful induction of specific humoral responses at the systemic and intestinal mucosa levels was observed when a prime-boost immunization scheme by s.c. and oral route was followed, as previously reported by other groups (Molina et al. 2005; Webster et al. 2006). A four-dose immunization schedule using the carrot-made HP6/TSOL18 vaccine was first employed to test its immunogenic properties. Thereafter, the protection induced by the vaccine orally applied was tested.

Orally administered carrot-made HP6/TSOL18 vaccine induced a significant reduction in parasite load against the experimental murine *T. crassiceps* cysticercosis. Considering the high recognition of anti-HP6/TSOL18 antibodies in several structures of *T. crassiceps* cysticerci (Fig. 5), the observed protection could be due in part to effector mechanisms of antibodies attached to the parasite, mediated either by antibody-dependent cytotoxicity or by cellular immunity. Remarkably, the observed protection was similar to that attained by s.c. administration of the *E. coli*-made antigen, reflecting a high immunogenicity of the plant-made HP6/TSOL18 antigen.

The availability of two oral vaccine candidates that promote either a humoral (HP6/TSOL18) or a cellular immunity (S3Pvac) can be taken into account to design new, more potent vaccination schedules. The combined use

of both vaccine candidates could offer a broader immune protection. Therefore, those parasites that could escape HP6/TSOL18-induced antibodies would be susceptible to the cellular immune response enhanced by S3Pvac. In addition, the expression system used in this study seems more effective than the papaya system previously used to express S3Pvac. For instance, a protein extract obtained from 40 mg of freeze-dried calli (from three lines processed in parallel) was used to immunize mice with S3Pvac, while the HP6/TSOL18 carrot-made vaccine consisted of a protein extract derived from 10 mg of a single carrot line. These differences justified the use of the carrot system to express the S3Pvac vaccine. It is also noteworthy that vaccine components from both papaya and carrot obviate the use of adjuvants to improve vaccine efficacy, required when *E. coli*-based recombinant GST-HP6/TSOL18 or synthetic S3Pvac was employed. Adjuvant compounds from plant cells may explain the high immunogenic activity of carrot-made vaccines, as suggested by several research groups (Mojica-Henshaw et al. 2003; Rosales-Mendoza and Salazar-González 2014).

Further experiments to assess the effect of additional booster doses to the immunization schedule, as well as the antigen stability in plant biomass, will be required. Our results are in agreement with several trials that have demonstrated the potential of carrot cells to deliver functional antigens by the oral route (Rosales-Mendoza and Tello-Olea 2015). These properties as delivery vehicle could be attributed to the plant cell-bioencapsulation that diminishes antigen degradation, while allowing a proper antigen release with a subsequent uptake by M cells.

In conclusion, carrot-made HP6/TSOL18 vaccine possesses immunogenic activity able to reduce the parasite load in mice, and thus, it is proposed as a promising candidate along with the S3Pvac antigens as an oral anti-cysticercosis vaccine (Hernández et al. 2007). Oral formulations composed by minimally processed plant biomass offer clear advantages, since starting materials for plant biomass production are inexpensive, and no cold-chain is required once plant cells are freeze-dried. Moreover, oral administration is safer and simplifies treatment.

Author contribution statement SRM and ES designed the research; EME, DGA, MH, JC, JSG, ARM, and GR generated and characterized the carrot lines; TG, EME, ES, GF and SRM analyzed data; ES, GF and SRM wrote the manuscript. All authors contributed to editing and approving the final version of the manuscript.

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