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Development of an Oral Anti-Cysticercosis Vaccine Delivered in Genetically Modified Embryogenic Callus of Papaya

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ABSTRACT

Vaccination of pigs may interrupt *Taenia solium* transmission by reducing the number of cysticerci, the precursors of adult intestinal tapeworms in humans. A novel preventive cost/effective polyvalent anti-cysticercosis vaccine expressed in papaya (S3Pvac-papaya) has been developed with the aim to increase its effectiveness at a reduced cost and offer an alternative oral route to increase the feasibility of its administration. Transgenic papaya plants have been developed with very few applications. This chapter reviews the advances in the development of an anti-cysticercosis vaccine expressed in papaya (*Carica papaya* L.). Sequences of the three peptides of the S3Pvac anti-cysticercosis vaccine named KETc7, KETc12 and KETc1 were used to transform papaya embryogenic callus. The integration of the transgene and their expression were confirmed in 19 clones by PCR, RT-PCR and real time RT-PCR. The protective capacity against the experimental murine cysticercosis of these transgenic lines was determined after subcutaneous immunization. The clones that induced the highest protective capacity were selected and used for oral immunization. A high level of protection, similar to that induced by subcutaneous immunization was observed. Interestingly, non-transformed papaya embryogenic callus also induced protection when subcutaneously administered albeit at a lower level. S3Pvac-papaya increased the levels of specific IgG antibodies in sera of immunized mice and also increased the CD4+ and CD8+ proliferative response in spleen cells and CD8+ in Peyer Patches. The obtained results point papaya cells as an advantageous delivery system of useful for the design of an effective, sustainable and affordable oral subunit vaccine against different pathogens.

Keywords: cysticercosis, S3Pvac vaccine, *Taenia crassiceps*, *Taenia solium*, transgenic papaya vaccination
Abbreviations: aa, amino acid; **EmpCL**, embryogenic papaya cells line; **ETgpC**, embryogenic transgenic papaya cell lines; **FWL**, fresh weight; **Hyg^R**, hygromicin resistance gene; **Km^R**, kanamycin resistance gene; **MS**, Murashige and Skoog medium; **NC**, neurocysticercosis; **PCR**, polymerase chain reaction; **PP**, Peyer Patches; **RT- PCR**, retrotranscriptase polymerase chain reaction; **rtRT-PCR**, real time retrotranscriptase polymerase chain reaction; **s.c.**, subcutaneous; **S3Pvac**, synthetic anti-cysticercosis vaccine

CONTENTS

INTRODUCTION

The use of transgenic plants as an expression system for the development of vaccines can potentially impact in future strategies of vaccination for the prevention of different diseases.

In particular, the expression of proteins in papaya represents a great potential for the production of molecules for therapeutic and vaccination purposes. Genetically transformed papaya callus can be regenerated and cultured *in vitro* to further establish cell suspensions, and it is possible to use this last culture system to massive produce oral vaccines at low cost. Moreover, the papaya system is highly amendable to genetic transformation (Cabrera Ponce *et al*. 1995) and the introduction of high copy number of the transgenes expressed at high levels (Cabrera Ponce *et al.* 1995; de la Fuente *et al*. 1997; Rossi *et al.* 1998; López-Gómez *et al.* 2008) represents another inherent advantage

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of papaya due to small genome size of only 372 megabases (Mb) and to the low gene numbers (Ming *et al.* 2008). In addition, papaya *per se* exhibits anti-parasitic properties of interest for the prevention of intestinal infections (Hammond *et al.* 1997; Stepek *et al.* 2004).

Embryogenic transgenic papaya clones (ETgpC) genetically transformed can be culture *in vitro* in long-terms keeping the expression of transgenes (selectable and reporter genes) nearly at the same level through years. Therefore it is achievable to be massively produced at low costs. These attractive characteristics led us to explore the development of a vaccine expressed in embryogenic papaya cell lines (EmpCL) in order to produce a new alternative for the expression of KETc1, KETc7 and KETc12 peptides that constitute the S3Pvac vaccine against porcine cysticercosis.

Taenia solium

Taeniasis and cysticercosis are infections caused by the adult and larval stages of *Taenia solium.* This parasite result in substantial economic condemnation due to the slaughter of cysticercotic swine, the obligatory intermediate hosts for the larval stage. Humans are the only final hosts for the tapeworm stage and become infected by ingesting raw or inadequately cooked cysticercotic meat. Mature tapeworms then produce thousands of eggs per day in the human host intestine, which are excreted and eventually consumed by the intermediate hosts (pigs) (Sciutto *et al.* 2000). Humans can also ingest *T. solium* tapeworm eggs, which can develop into the larval stage, causing cysticercosis. When the parasite lodged in the central nervous system causes neurocysticercosis (NC), the most frequent, severe and even fatal form of the infection in humans develops (Fleury *et al.* 2004). The great majority of cases occur in developing countries (Sciutto *et al.* 2000); however, it is considered as an emerging disease in the developed regions due to immigration (Schantz *et al.* 1998). Extra central nervous system infections are also frequently reported in Asia and Africa (Willingham *et al.* 2003) but not in America (Cruz *et al.* 1994). Prevalence rates vary in the endemic countries but are estimated at less than 1:1000 for human taeniasis (tapeworm carriers), 1-10% for human cysticercosis (Garcia *et al.* 1998), and up to 20-40% for pig cysticercosis (Morales *et al.* 2006). Variations of the rates depend on sample design (e.g. rural vs. urban), form of detection (i.e., radiological vs. serologic) and risk factors (i.e., family history of taeniasis). In other studies, human NC was found in 1-5% of a large autopsy series in Mexico (Rabiela 1972; Villagrán and Olvera 1988), which accounted for about 10% in rural populations according to two epidemiological studies based on computed tomography (CT) scan ((Fleury *et al.* 2003, 2006) and is the most frequent cause of epilepsy in neurological patients (Del Brutto *et al.* 1993).

There is a clear need to control *T. solium* because it seriously affects human health and animal husbandry in undeveloped and developed countries. The high stability of the life-cycle point to the need for the implementation of simultaneous and sustained multiple interventions.

CYSTICERCOSIS CONTROL

Cestodiasis represents a frequent health problem widespread in developed and developing countries. Taeniasis/cysticercosis caused by *T. solium* are the main cestodiasis affecting human health and animal production.

In spite of the high prevalence of *T. solium* cysticercosis and its impact on human and veterinary health and economy, their eradication remains a major challenge, particularly in undeveloped countries where the cestodes' life-cycles are firmly established in their social, cultural and economic context. Intervention programs aimed (R2) at improving sanitary conditions; health education; detection and treatment of tapeworm carriers, and the treatment of the intermediate hosts have been proposed and successfully tested locally (Sarti *et al.* 1997; Roman *et al.* 2000; Sarti *et al.* 2000; Boa *et al.* 2003; Eddi *et al.* 2003; Larralde and Sciutto 2006). However, these limited actions are not enough to achieve wide and long-term control due to the complex economic and social networks that underlie cestode transmission (Engels *et al.* 2003; Pawlowski *et al.* 2005). Increased resistance against the establishment of the larval stage in the intermediate hosts, by improving the specific host immune response through vaccination is a strategy previously considered but not employed yet. Indeed, extensive reports indicate that the development of an effective vaccine against cysticercosis is a feasible goal, a recombinant vaccine was already developed against *T. solium* cysticercosis (Morales *et al.* 2008) and several candidates experimentally tested have also been reported (Sciutto *et al.* 1990; Nascimento *et al.* 1995; Molinari *et al.* 1997; Lightowlers *et al.* 1999; Plancarte *et al.* 1999; Lightowlers *et al.* 2000; Cai *et al.* 2001; Huerta *et al.* 2001; Sciutto *et al.* 2002; Flisser *et al.* 2004; Guo *et al.* 2004; Manoutcharian *et al.* 2004; Gonzalez *et al.* 2005; Sciutto *et al.* 2008). However, all these vaccines (R2) were parentally administered which is a paramount limitation for their application in a nationwide and sustained control program. The low feasibility of its massive and inexpensive production and the high costs and logistic difficulties of the administration of an injected vaccine are the main reasons for the absence of vaccination programs worldwide.

Thus, the development of an effective low-cost polyvalent oral vaccine that could be simply and extensively administered for the prevention of cysticercosis by improving the mucosal and systemic specific immunity through oral vaccination, using the set of 3 highly protective peptides previously identified (Manoutcharian *et al*. 1996), represents an important tool toward the control of this parasitosis.

Effective vaccine candidates against cysticercosis

The vaccines against cestodiasis caused by the metacestode stage have consistently proven effective under experimental conditions. Indeed, several effective vaccine candidates have been developed since the pioneering work of Rickard and White (1976) against the larval phase of *Taenia ovis*. There are currently a number of reports on successful vaccination trials against metacestode diseases caused by the larval stage of *T. solium* (Nascimento *et al.* 1995; Molinari *et al.* 1997; Plancarte *et al.* 1999; Huerta *et al.* 2001; Flisser *et al.* 2004; Manoutcharian *et al.* 2004).

S3Pvac anti-cysticercosis vaccine

An experimental murine model of cysticercosis caused by *Taenia crassiceps* was used to identify promising vaccine candidates against *T. solium* pig cysticercosis. This model has proven to be a fast inexpensive and effective first screening to identify antigens of interest for vaccination and for testing different vaccination approaches (Sciutto *et al.* 2007). Three protective peptides against cysticercosis were identified in a cDNA *T. crassiceps* library (Manoutcharian *et al.* 1996). The three peptides that constitute the S3Pvac anti-cysticercosis vaccine are of 8 (KETc12), 12 (KETc1) and 110 amino acids long (KETc7) (Rosas *et al.* 1998; Toledo *et al.* 1999, 2001). Two protective peptides were identified in KETc7, GK1 of 18 aminoacids (aa) and PT1 of 10 aa long (Manoutcharian *et al.* 1999).

These peptides belong to native antigens that are present in the different developmental stages of *T. crassiceps.* The respective homologous antigens were also found in *T. solium* (Rosas *et al.* 1998; Toledo *et al.* 1999, 2001) in different anatomical structures of the cysticerci, the eggs and the adult tapeworm. Synthetically expressed S3Pvac was tested in the field in communities of the State of Puebla, Mexico (Huerta *et al.* 2001). Vaccination reduced the number of infected pigs by 50% and 98% of the amount of *T. solium* cysticerci and, therefore, the amount of cysticerci potentially able to transform into tapeworms. Upon reevaluation in a different community of the State of Morelos simi-

lar results were obtained (Sciutto *et al.* 2007). Thus, the effectiveness of S3Pvac in preventing naturally acquired porcine cysticercosis has been demonstrated.

More recently, a second version of this synthetic vaccine recombinantly expressed in filamentous phages (S3Pvac-phage) was produced and tested in the field in a randomized trial that included 1,047 rural pigs in 16 villages of Central Mexico (Morales *et al*. 2008). At 5 to 27 months of age, 331 pigs (197 vaccinated/134 controls) were inspected at necropsy. Vaccination reduced the frequency of tongue cysticercosis by 70% and, based on necropsy, 54% of muscle-cysticercosis and by 87% of the number of cysticerci (Morales *et al.* 2008).

Anti-cysticercosis oral vaccine

Many pathogens, among them helminths, enter to the human body through the mucosal surfaces; thus, mucosal immunization seems to be a promising strategy to prevent infections. Moreover, the needle-free oral vaccines are by far the most attractive method of vaccine delivery, which also avoid costly logistics problems making them suitable for massive immunization programs. The cost of vaccine production is an important issue to consider in the design of vaccines against pig cysticercosis because the low cost of the host to be protected, the difficulties to hold reared free pigs and because it will be applied amongst the poorest sectors of non-developed countries. With respect to this, the expression of the protective peptides using cell cultures of transformed papaya cells is a suitable delivery system for low-cost massive production overridden the expensive step of antigen purification and the costly artificial antigen encapsulation technologies. Furthermore, plant cell walls can protect antigens of further degradation in the digestive tract enabling them to reach the Gut Associated Lymphoid Tissue (GALT) (Mason 2002; Walmsley and Arntzen 2003).

S3Pvac anti-cysticercosis oral vaccine expressed in embryogenic papaya callus

Transgenic plants are ideal systems to produce oral vaccines since the walls of the plant cell may protect antigenic proteins from decay in the stomach enabling them for effective presentation to mucosal-associated lymphoid tissues (MALT) (Walmsley and Arntzen 2003).

1. Genetic transformation

Seeds of papaya 'Maradol' Tabasco (*Carica papaya* L.) were isolated from young green fruits and used a source of zygotic embryos for the induction of embryogenic papaya cell lines (EmpCL) according to Cabrera-Ponce *et al.* (1995).

EmpCL at the globular stage (**Fig. 1A**) was co-transformed using the particle bombardment system PDS-1000/He (BioRAD) and the Sanford *et al*. (1991) and Tomes *et al*. (1995) protocol with some modifications (Cabrera-Ponce *et al*. 1995, 1997). Briefly, the pWRG1515 plasmid containing gus-A (β-glucuronidase reporter gene) and the hygromicin resistance gene (Hyg^R) (Christou *et al.* 1992) was employed with the pUI 235-5.1 vector containing the KETc1.6His or KETc12.6His or KETc7 insert and the kanamycin resistance gene (Km^R). Calli were bombarded once at P=800 psi. Selection of embryogenic transgenic papaya clones (ETgpC) was done according to Cabrera-Ponce *et al.* (1995), with modifications. Hygromicin (50 mg/L) and kanamycin (100 mg/L) were used together as selectable markers. Two to three subcultures (monthly) on this medium selection were applied and the ETgpC, resistant to Hyg^R and Km^R were propagated and progressively numbered, adding an initial p for papaya, e.g. $pKETc7_{21}$ (Hernández *et al.* 2007). Embryogenic papaya embryos of some ETgpC were incubated with X-GLUC (5-bromo-4-chloro-3-indolyl β-D-glucuronic acid) a few minutes until 24 hours at room temperature to detect the transitory expression of the reporter gene (**Fig. 1B**).

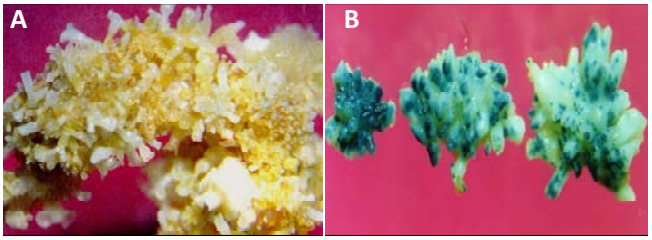

Fig. 1 Aspect of embryogenic callus cultures. EmpCL at the globular stage were used for genetic transformation (**A**). Transient expression of β-glucoronidase in ETgpC (**B**).

2. Genetic and immunological ETgpC characterization

The presence of the transgene and the peptide expression in each of the ETgpC, were determined by PCR, RT-PCR and rtRT-PCR. Different immunological procedures (HPLC, Western blot, ELISA) were used to identify the vaccine peptides in the different clones but all failed to detect the peptides. Considering that modifications in the vaccine epitopes expressed in ETgpC could underlie the absence of recognition, sera from vaccinated mice with the ETgpC were used to directly expose to the parasite surface in search of specific binding. Immunohistochemical studies, revealed the presence of specific antibodies that recognized the native peptides (KETc1 and KETc12) in the tegument and parenchyma regions, respectively. Low levels of KETc7 were detected in the parenchyma tissue but higher levels were expressed in the cysticerci tegument. No recognition was obtained when sera from mice immunized with EmpCL were employed (Hernández *et al.* 2007).

Those ETgpC which express the vaccine transcripts at higher levels were selected and *in vitro* propagated. Soluble extract were prepared and the total protein determined (Hernández *et al*. 2007).

Identification of the protective capacity of the selected ETgpC against experimental *Taenia crassiceps* murine cysticercosis

Using the procedure previously described several clones were produced. **Fig. 1** shows the macroscopic aspect of the cultures. We first selected the most promising according with the level of expression of the respective transcript. The selected clones named $pKETc1_{1, 2, 3, 5, 9, 11, 12, 25, 41}$, $pKETc7_{21, 23}$ and $pKETc12_{6, 7}$ were propagated under controlled conditions by *in vitro* subculture following the procedure previously described (Cabrera-Ponce *et al.* 1995). The protection induced by subcutaneous (s.c.) immunization using a soluble extract prepared from clones was evaluated (Hernández *et al.* 2007). Furthermore, the ETgpC clone named $pKETc7_{23}$ was extensively employed to identify the most adequate conditions for vaccination. As shown in **Table 1**, a soluble extract was employed for oral and for s.c. immunization and similar level-extent of protection were observed. In addition, a suspension of the transgenic line was also tested in equivalent amount of total proteins. Briefly, the suspension was prepared by grinding the ETgpC embryogenic papaya callus from EmpCL and ETgpC in liquid nitrogen followed by freeze-drying. The resulting pulverized tissue was stored at -80°C until used (Molina *et al*. 2005).

Unexpectedly no significant differences in the induced protection were observed using soluble extract at doses of 200 to 1000 μg or the equivalent of 10 to 40 mg of cell suspension. In addition, it is remarkable the high level of induced protection without the need of purification, without the use of any adjuvant and with only one booster, a finding that increases the relevance of papaya as a delivery system.

Oral immunization with EmpCL did not modify the expected parasite load. In contrast, high level protection was induced by s.c. immunization with the EmpCL itself. This protection is induced by a non-specific response since sera

Table 1 Effective protection against experimental murine cysticercosis with soluble extract or a cell suspension of pKETc7$_{23}$ ETgpC.

Route of immunization	Individual number of cysticerci	Median [95% confidence limits]	Protection (%)	Tpm (%)
Non-immunized:	1, 56, 135, 153, 203, 224, 245[a]	153 [62.5–228.4]		0[a]
S.c. Immunization				
EmpCL	0, 1, 3, 10, 13, 25, 46, 54, 114[b]	13 [0.96–58.2]	79.5[a]	11[a]
pKETc7$_{23}$ (soluble extract)				
200 µg/mouse	0, 0, 0, 0, 0, 0, 0, 1, 3, 12, 66[c]	0 [−9.2–24.5]	94.8[a]	63[b]
1000 µg/mouse	0, 0, 0, 0, 0, 2, 8, 73, 241, 249[b,c]	1 [−15.3–129.9]	60.5[a]	50[b]
Non-immunized:	†78, 125, 172, 173, 130, 227, 434[a]	172 [83.2 – 299.3]		0[a]
Oral Immunization				
pKETc7$_{23}$ (suspension)				
10 mg/mouse	†0, 1, 5, 7, 11, 36, 88, 95, 113[b]	11 [4.2 - 74.9]	79.2[a]	11[b]
40 mg/mouse	†0, 0, 0, 0, 47, 61, 70, 105[b]	23.5 [0.98 - 69.7]	81.5[a]	50[b]
pKETc7$_{23}$ (soluble extract)				
50 µg/mouse	0, 1, 1, 3, 37, 46, 65, 137, 142	37 [4.2-91.7]	74.8[a]	11[b]
200 µg/mouse	0, 5, 7, 9, 14, 55, 60	9 [-1.7-44.6]	88.8[a]	14[b]

Cysticerci were recovered 40 days after challenged. Tpm (totally protected mice): number of mice bearing zero cysticerci divided by the number of mice tested in the group×100. Different superscript letters indicate the statistically significant differences ($P < 0.05$)
Data of subcutaneous immunization were taken from Hernández *et al*. (2007). Data from oral immunization have not been previously published.

from EmpCL immunized mice did not recognize the cysticerci according to the immunohistochemical analysis (Hernández *et al*. 2007). It is possible that factors related to an exacerbated innate response could be involved. Considering the magnitude of this non-specific response, further studies against this and other parasitic disease are being performed. Nevertheless, this result adds additional interest for using at papaya as a delivery system in parenteral vaccination.

Specific immunity elicited by oral immunization with S3Pvac-papaya

The systemic and local immunity elicited by oral vaccination using the ETgpC were analyzed using the pKETc1$_9$ clone. As **Fig. 2** shows, oral immunization with this clone elicited in sera detectable levels of specific IgG and IgA determined by ELISA following the procedures previously described (Rosas *et al*. 2006). **Fig. 3** illustrates some of the changes in the lymphocyte cell population that were also induced by vaccination: an increased CD4+ and CD8+ proliferative response in spleen cells and CD8+ in Peyer Patches (PP).

Stability of the S3Pvac-papaya and scale-up production

The expression of the ETgpC has been tested during the last four years since its production. **Fig. 4** shows the presence of the transgene detected by PCR in the selected highly protected ETgpC. As it shows the respective inserts of the expected sizes were conserved after 4 years of *in vitro* maintenance.

Cell suspension cultures of the most promising ETgpC (pKETc12$_6$, pKETc1$_9$, pKETc7$_{23}$) expressing KETc12.6 His, KETc1.6His and KETc7 peptides are being initiated in Erlenmeyer flasks and grown in agitation in Murashige and Skoog medium (MS) (Murashige and Skoog 1962) supplemented with plant growth regulators as described by Cabrera-Ponce *et al.* (1995) in a batch mode over 25 days. In order to obtain higher biomasses and to increase the productivity levels, the scale-up of the cell suspensions lines, are actually being implemented in 2 and 10-L draught-tube internal-loop airlift reactors. In such units 20-day-old cultures grown in 1-L flask are used as inoculum (10 g FWL^{-1}). Three to four-week-old batch-suspension cultures in bioreactors are growing in MS nutrient medium. During cultivation, conditions for reactors are 26-27°C temperature and uniform white light (8-10 µmol s^{-1} m^{-2}). Air is supplied at various flow rates from 0.1- to 0.01 vvm (volume of air per liquid volume min $^{-1}$), according to cell growth and in order to maintain culture homogeneity. Kinetic parameters on growth and peptides productivity will be obtained.

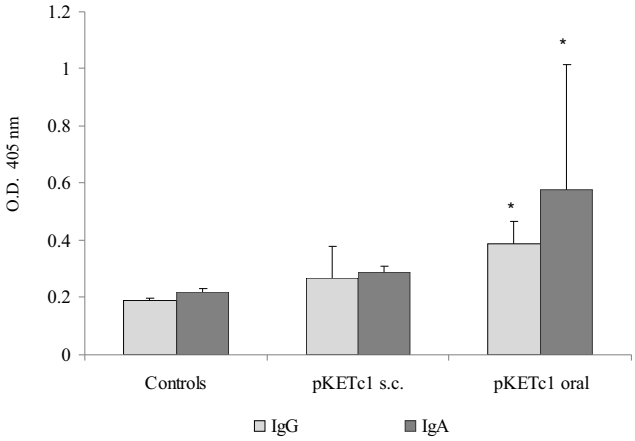

Fig. 2 Antibody response induced by s.c. or oral immunization with the pKETc1$_9$ ETgpC. Levels of antibodies against KETc1 were determined in duplicate in each of 7 mice per group 15 days after the last immunization. Mice were s.c or orally immunized with the pKETc1$_9$ ETgpC. Results are expressed as mean ± SD of OD. *Significant increased levels of antibodies in vaccinated mice ($P < 0.05$) using the Mann-Whitney test.

CONCLUSIONS AND PERSPECTIVES

Papaya has proved to be an effective system using microparticle bombardment (biobalistics). Genetic transformation of embryogenic callus of papaya (*Carica papaya* L.) mediated by particle bombardment has been shown to be a useful system to express transgenes more efficiently compared to model species like tobacco, potato or *Arabidopsis* (Cabrera Ponce *et al.* 1995; de la Fuente *et al*. 1997; Rossi *et al.* 1998; López-Gomez *et al.* 2008). Considering this embryogenic callus were genetically transformed with three peptides (KETc7, KETc1 and KETc12) in order to develop an oral anti-cysticercosis vaccine. The effective protection induced without any undesirable effect point to its possible use for cysticercosis prevention. The anti-cysticercosis vaccine is being developed towards commercial application. The high efficiency and the innocuity and the non invasive form of administration of these vaccines led us to consider extending its use against human cysticercosis.

The evaluation of the oral vaccine in the field under natural conditions of transmission is an essential study that has to be done in the near future to validate its use.

One limit of the available vaccine is the low amount of peptides constitutively expressed in ETgpC (Hernández *et al.* 2007) that could be overridden by the scale-up production.

The above mentioned major advantages of the *in vitro*

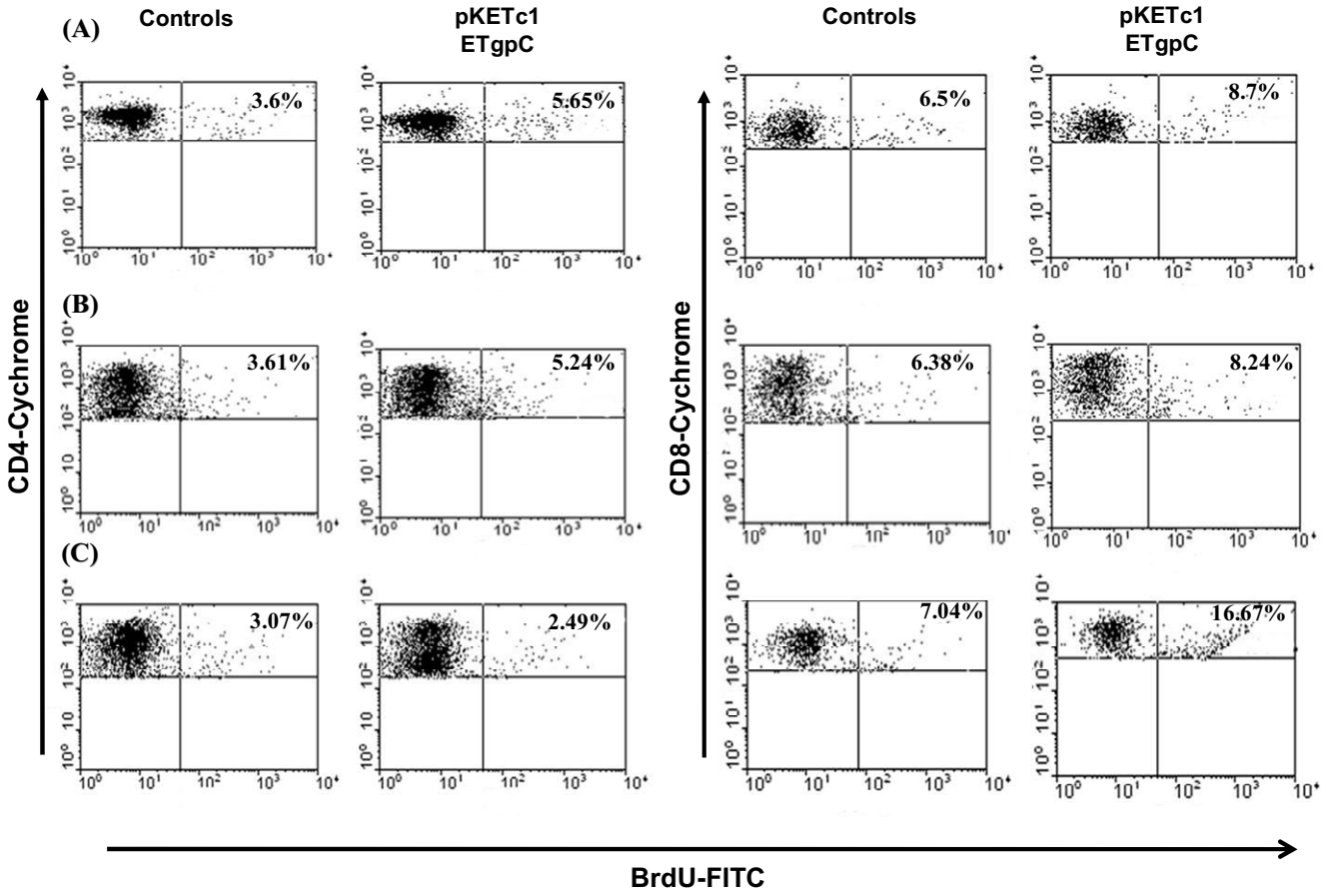

Fig. 3 CD4+ and CD8+ T cell proliferation induced by s.c or oral immunization with the pKETc1$_9$ ETgpC. Fresh CD4+ or CD8+ spleen and lymphoid PP cells from pooled from three mice pKETc1$_9$ ETgpC immunized or non-immunized (naïve) were evaluated for BrdU incorporation in proliferating cells by cell cytometry (FACs). The percentage of positive cells in each quadrant is shown. CD4+ and CD8+ proliferation in spleen cells of mice subcutaneously immunized (**A**) and in spleen (**B**) and PP (**C**) cells from mice orally immunized.

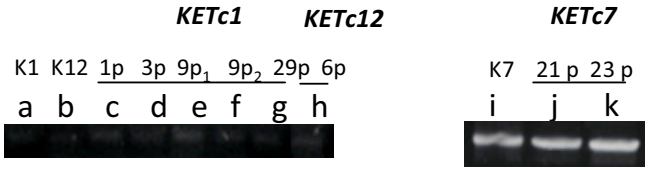

Fig. 4 Detection of the transgene in transgenic ETgpC. Analysis of ETgpC revealed the presence of the KETc1.6His, KETc12.6His and KETc7 transgenes by PCR. Lane a, b and i: pUI235-5.1 vector with KETc1.6His, KETc12.6 His and KETc7, respectively. Lanes c, d, e, f , g are different ETgpC transformed with the KETc1.6His insert, lane h shows an ETgpC transformed with the KETc12.6His insert and j and k are different ETgpC transformed with the KETc7 insert. It is relevant to remark that the ETgpC have been *in vitro* reproduced from embryogenic callus since the last four years.

obtained papaya cultures (callus and cell suspensions) strongly indicate the possibility of a design for an up scaling process for further biotechnological exploration as well as for future commercial purposes. The feasibility of cell suspension growth for the sustainable and controlled production of the S3Pvac-papaya vaccine on a large-scale basis is actually being explored in airlift bioreactor systems.

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