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# Alga-based HPV16 E7 vaccine elicits specific immune response in mice

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**ABSTRACT**

*Recent development of antigen-expressing plant cells indicated their potential in low-cost vaccines production for large scale vaccination campaigns in developing countries. In this study, we engineered the chloroplasts of unicellular alga Chlamydomonas reinhardtii for the expression of human papillomavirus 16 oncoprotein E7, mutated for low oncogenicity and fused with aadA gene conferring spectinomycin resistance. The potential of transplastomic algae extracts as therapeutic vaccine against oncogenic papillomavirus infection was studied. Fusion protein could be detected in transplastomic algae together with its degradation products. Analysis of immune reactions induced in mice by vaccination with algae extracts showed high production of E7-specific antibodies, but low activation of E7-specific $CD8^+$ cells.*

**Key words:** *Chlamydomonas reinhardtii*, chloroplast transformation, human papillomaviruses, E7 oncogene.

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## INTRODUCTION

Genital infection caused by human papillomavirus (HPV) is the most common sexually transmitted viral disease worldwide. The so-called "high risk" or oncogenic HPVs have been found to be associated with cervical carcinomas, the second biggest cause of female cancer mortality worldwide [1, 2]. Recent introduction of prophylactic vaccines that prevent infection by oncogenic HPVs represents a major success in fighting this disease in countries, where large-scale vaccination campaigns could be organized [3, 4]. On the other hand, therapeutic vaccines stimulating cell-mediated immunity against viral nonstructural proteins so as to eliminate infected cells are yet not available in clinical praxis, even though clinical trials were reported several years ago [5]. Such vaccines are often targeted against viral oncoprotein E7 that inactivates cellular antitumor protein pRb. Because E7 is only a weak immunogen, fusions with other proteins are being constructed and some of them proved to be highly immunogenic in mouse model [6].

Vaccine production is an industrial process requiring substantial investment [7] which often limits availability of latest-generation vaccines in developing countries. During the past 15 years, however, antigen-expressing crops developed by plant biotechnology methods have become a cost-effective alternative for industrial vaccine production as they could be produced locally with only basic agricultural knowledge. Transplastomic plants expressing antigens from engineered chloroplast genome are especially recommended as environmentally friendly, genetically modified organisms (GMO), showing minimal out-crossing of transgenes to related weeds or crops. Because of highly polyploid chloroplast genome, transformation of chloroplasts permits the introduction of many copies of a foreign gene per a plant cell, generating high levels of the foreign protein.

Among plant cells often promoted as a vaccine-factory, green alga *Chlamydomonas reinhardtii* has a special position as it unites several important characteristics for an ideal plant vaccine producer [8]: It can by cultivated in a contained reactor at low cost with biomass increase much quicker than edible plants can yield. The entire alga biomass is suitable for vaccination and no GMO waste material is produced. *Chlamydomonas*-specific molecular toolbox enables straightforward construction of homoplastic *C. reinhardtii* with high-level antigen expression. Antigen-expressing algae can by lyophilized and then stored for over 1.5 years without cooling at any stage [9].

In this study, transplastomic *C. reinhardtii* cells were prepared expressing fusion of modified HPV16 *E7* gene with *aadA* gene conferring spectinomycin resistance. Mice were vaccinated with transplastomic algal extracts and their immunogenic properties were studied.

## MATERIALS AND METHODS

### *HPV E7 fusion gene and chloroplast expression cassette*

All DNA manipulations were carried out essentially as described by [10]. Genes were commercially synthesized by GeneArt, Regensburg, Germany. Human papillomavirus type 16 *E7* gene was designed and de novo synthesized with mutations improving E7 immunization properties. Synthetic *E7GGGp* gene was fused "in frame" with *aadA* gene conferring spectinomycin resistance in pFaadAII chloroplast vector [11] and the resulting *E7GGGp/aadA* gene was integrated between *C. reinhardtii* rbcL SD sequence and rbcL termination sequence, with start codon of *E7GGGp/aadA* in the position of the original rbcL gene [12]. Finally, tobacco chloroplast 16S rDNA promoter from chloroplast vector pFaadAII [10] was integrated to drive expression of the cassette *E7GGGp/aadA*.

### *Production of E7GGGp/aadA in bacteria*

BamHI fragment carrying the expression cassette was recloned in pET-22b expression vector (Merck). Both original plasmid constructs in pGA4 (GeneArt) and recloned cassettes were transformed in Escherichia coli BL21(DE3) cells and selected clones were grown in Luria-Bertani medium supplemented with 100 μg/ml ampicilin to an $OD_{600}$ 0.6 before T7 promoter driven E7GGGp/aadA production was induced for 1 hour by addition of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG).

Cells (1 ml) were harvested by centrifugation and 25 μl of B-PERII (Pierce) extraction buffer was added, cells resuspended in B-PERII and lysed at room temperature.

### *Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of crude bacterial and algal extracts and western blotting of E7 protein*

Electrophoresis was performed in OWL 10 x 10 cm elfo unit P8DS according to Laemmli [13], with 4% PAGE stacking gel and 8% PAGE resolving gel, and proteins were detected by staining with SERVA blue G. For western blotting, proteins separated by SDS-PAGE were electroblotted onto a HYBOND-ECL nitrocellulose membrane (Amersham Biosciences, Little Chalfont, UK) and treated in blocking buffer as described [14]. After decanting the blocking buffer, the membrane was incubated with anti-E7 antibodies and subsequently, with secondary peroxidase-labeled antibodies (Amersham Biosciences). Blots were stained using the ECL Plus system (Amersham Biosciences).

### *Chloroplast vector*

Intergeneric sequence 46756 – 47750 from *C. reinhardtii* chloroplast IR region was synthesized and cloned in pMK-RQ plasmid (GeneArt). *E7GGGp/aadA* expression cassette was integrated in a unique BamHI site in the centre of the synthetic fragment of *C. reinhardtii* chloroplast IT region (Fig.1).

### **Chlamydomonas reinhardtii** *strain, transformation and growth conditions*

All transformations were carried out on *Chlamydomonas reinhardtii* strain CC-125 obtained from the *Chlamydomonas* stock Center (Duke University, Durham, NC, USA). Cells were grown in TAP medium [15] in liquid culture and collected in exponential growth phase (cca $5 \times 10^6$ cells/ml). After centrifugation, peleted cells were resuspended in fresh TAP medium in density of $0.5 \times 10^9$ cells/ml and 300 μl of suspension was evenly spread on 0.2 μm membrane filter of 47 mm diameter and placed on solid TAP medium supplemented with 2% agar. *Chlamydomonas* cells were then bombarded in PDS-1000/He Particle Delivery System with gold 1.0 μm particles coated with circular or linearized plasmid DNA using 1100 psi helium pressure and 6 cm distance of target cells from stopping screen. Total of 76 agar plates were bombarded. After 36 hours cultivation in dim light and 22°C they were transferred to selection media (TAP + spectinomycin 150 mg/l) and later transferred to fresh selection media every 7 days. Homoplastic *C. reinhardtii* strains were generated by sequentially streaking the clones ten times on spectinomycin-selective TAP agar plates.

***PCR and Southern blot analyses***

PCR assays were performed according to [16]. The use of P1 (5'-GAA GCG GTT ATC GCC GAA G-3') and P2 (5'-TTA TTT GCC AAC TAC CTT AGT GAT C-3') primers [17] resulted in amplification of an *aadA* gene fragment of 787 bp. The PCR profile was as follows: 45 s denaturation at 94°C, 30 s annealing at 62°C, 3 min extension at 72°C for a total of 35 cycles followed with final extension for 10 min at 72°C.

Genomic DNA (15 µg) isolated from algal extracts according to [18] was used for Southern blot analyses. DNA was digested with *Sac*I restriction enzyme, electrophoresed overnight in 1% agarose gel with TBE buffer and transferred onto nylon Hybond-$N^+$ (GE Healthcare, UK) membrane. Hybridization procedure according to [19] was used and 787 bp DNA fragment derived from *aadA* gene served as probe. Probes was labeled with [$\alpha$-$^{32}$P]dCTP ($1.11 \times 10^8$ MBq/mmol) using a random priming kit Rediprime™ II (GE Healthcare, UK) and the membranes were scanned using the phosphoimager Typhoon system (Amersham Biosciences, UK) after 5h exposure.

***Immunization of mice and detection of induced E7 antibodies***

Six- to eight-week-old female C57BL/6 mice (H-2b) (Charles River, Germany) maintained under standard conditions at the Center for Experimental Biomodels (Charles University, Prague) were immunized three times at three-week intervals with 3 mg of algal lysate. The lysate in 200 µl of Freud´s adjuvans or in PBS buffer with 25 µg of immunostimulatory CpG motifs (ODN 1826: TCCATGACGTTCCTGACGTT) was administered by s.c. injection. Two weeks after the last immunization dose sera of immunized mice were collected and pools of lymphocytes were isolated from splenocytes (three mice per group) using Histopaque-1077 (Sigma) and analysed by an ELISA and ELISPOT assays. The experiments proposed and performed in this project have been approved by the Commission for work with laboratory animals; animal experiments were performed by workers with certificates for work with laboratory animals.

***ELISPOT assay***

MultiScreen 96-well filtration plates (Millipore, Molsheim, France) were coated with 10ug/ml of rat anti-mouse IFN-γ antibody (BD Biosciences Pharmingen, San Diego, CA) or with anti-mouse IL-4 (concentration as recommended by the manufacturer, eBioscience, San Diego, CA) in 50 µl of PBS and incubated overnight at 4 °C. Lymphocytes were added to the plate ($10^6$/well) and incubated at 37°C in 5% $CO_2$. IFN-γ plates were incubated 20h either with or without 0.1 ug/ml HPV16 E7 peptide RAHYNIVTF (49-57aa). IL-4 plates were incubated 48h with or without 10 µg/ml HPV16 E7 44-62aa peptide. The cells were removed by two washes with deionized water and three washes with PBS–0.05% Tween 20, each washing taking 5min. Then, 4 µg/ml of biotinylated rat anti-mouse IFN-γ antibody (BD Biosciences Pharmingen) and rat anti-mouse IL-4 antibody (concentration as recommended by the manufacturer, eBioscience) in 50 µl PBS were added per well and cultivated at 4°C overnight. The wells were washed three times with PBS–0.05% Tween 20 for 2 min. and incubated for 30 min. with 50 µl of 1:100 dilution of streptavidin-horseradish peroxidase (BD Biosciences Pharmingen) in PBS at 37°C in 5% $CO_2$. After washing four times with PBS–0.05% Tween 20 for 2 min., followed by two washing steps with PBS alone, the spots were developed by adding 50 µl of an AEC chromogen and AEC substrate mixture (AEC Substrate Set, BD Biosciences Pharmingen) and incubation at room temperature for 5–15 min. They were counted by a S5 UV Immunospot analyzer (CTL).

***GST fusion protein***

Viral antigens were expressed with pGEX vectors (Amersham, UK )in *Escherichia coli*- strain BL-21 as double fusion proteins with N-terminal GST and a C-terminal peptide (tag) consisting of the 11 C-terminal amino acids from the large T antigen of simian virus 40. The expression constructs for E7 of HPV type 16 as GST fusion protein have been described [20]. A fusion protein consisting of GST and tag without intervening viral antigen was used for background determinations. Overexpression of GST fusion proteins was induced by addition of 250 µmol/L isopropyl-β-d-thiogalactoside to the bacterial culture. Bacterial cells were harvested by centrifugation, and the cells were lysed in a high-pressure homogenizer (Avestin, Canada). The lysate was cleared from insoluble components by centrifugation (30 min. at 4°C and 30 000 rpm), and the supernatant was mixed 1:1 with glycerol and stored at -20°C [21].

***GST capture ELISA***

Polysorp 96-well plastic plates (Nunc, Denmark) were coated overnight at 4°C with 200 µg/well of glutathione casein in 50 mM carbonate buffer, pH 9.6. Wells were incubated for 1 h at 37°C with 200 µl of blocking buffer (0.2% casein, Sigma, Germany) in PBS, 0.05% Tween 20 followed by incubation with the cleared lysates from E. coli expressing GST-E7-tag protein diluted in blocking buffer to 0.25 mg/ml total lysate protein (saturating amount) for 1 h at 37°C. Unbound material was then washed away. Mouse sera to be assayed for E7 antibodies were diluted 1:50 in blocking buffer containing 0.25 mg/ml total lysate protein from the GST-tag-transformed E. coli. Antigen-

coated ELISA plates were then incubated for 1 h at 37°C with 100 ml/well of diluted serum. After washing the plates, bound mouse antibodies were detected by sheep anti-mouse IgG conjugated to HRP (Amersham, UK) in dilution 1:2000 in blocking buffer for 1 h at 37°C. The color reaction using tetramethylbenzidine (Sigma, Germany) as substrate was started (10ug/ml in 0.1 M NaAcetate, pH 6.0, 0.003% $H_2O_2$, 100 µl/well). Reaction was stopped by adding 50 µl of 1 M sulfuric acid per well and the absorbance at 450 nm was measured in a Tecan (Austria) automated plate reader.

## RESULTS

***Design of transgenic C. reinhardtii engineered for the expression of HPV16 E7 antigen in chloroplasts***

To adapt the E7 oncoprotein for immunization, a synthetic gene was prepared with optimized codon bias for higher expression in chloroplasts and three amino acid substitutions in Rb-binding site that are known to reduce E7 oncogenicity but not immunogenicity [22]. Fused with marker gene *aadA* and equipped with chloroplast-expression signals, the selectable expression cassette *E7GGGp/aadA* was integrated between 500b long, synthetic flanking sequences derived from IR region of *C. reihardtii* chloroplast. Both circular and unique SacI site linearized vectors were used for *C. reinhardtii* CC-137 chloroplast transformation.

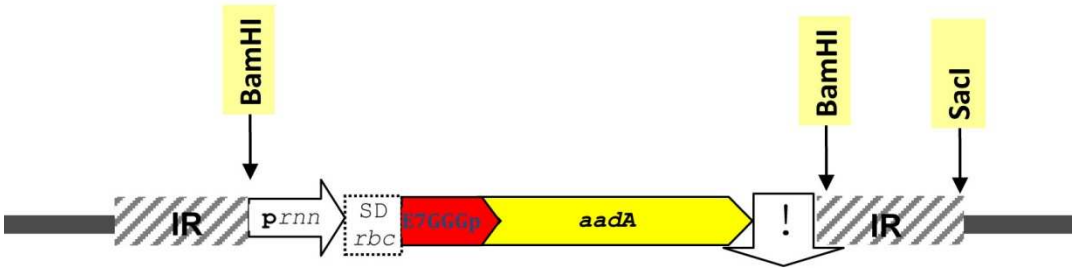


**Fig. 1. Chlamydomonas chloroplast transformation vector.**

p*rrn* = tobacco 16S rRNA gene promoter; SD*rbcL* = Shine-Dalgarno sequence of *C. reinhardtii rbcL* gene; ! = termination sequence of *C. reinhardtii rbcL* gene; *E7GGGp* = *E7GGG* oncogene of human papillomavirus strain 16, codon optimized; *aadA* = aminoglycoside adenylyltransferase gene.

***E7GGGp/aadA expression in bacteria***

To determine if the semi-synthetic fusion cassette is capable of producing functional pHPV16 E7 protein, we examined cell lysates prepared from bacterial cells transformed with either the original chloroplast vector (Fig. 1, expression driven by chloroplast 16S rRNA promoter) or with recloned cassette in pET-22b (expression driven by T7 promoter) that is known as one of the most efficient systems for producing recombinant proteins [23]. Western blot analysis revealed high expression fusion protein E7GGGp/aadA in pET-22b transformed bacteria but undetectable with the original chloroplast vector (Fig. 2).

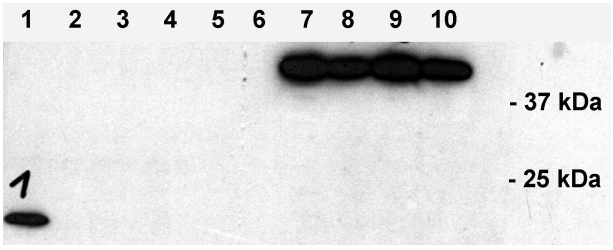


**Fig. 2. Western blot of extracts from bacterial clones expressing E7GGGp/aadA cassette.**

5 µl of bacterial extract was applied. Lane 1, standard E7; lanes 2 – 6, clones carrying chloroplast expression vector; lanes 7 – 10, clones carrying expression cassette in pET-22b vector.

***C. reinhardtii* chloroplast transformation and Southern blot analysis of transformants.**

Cells were transformed with both circular and linear chloroplast vector conferring resistance to spectinomycin and 194 primary transformants were obtained. Screening by PCR revealed only 25 positive transformants, all obtained with circular vector. Positive clones were taken through additional rounds of selection to isolate homoplastic lines that were analyzed by Southern blot (Fig. 3).

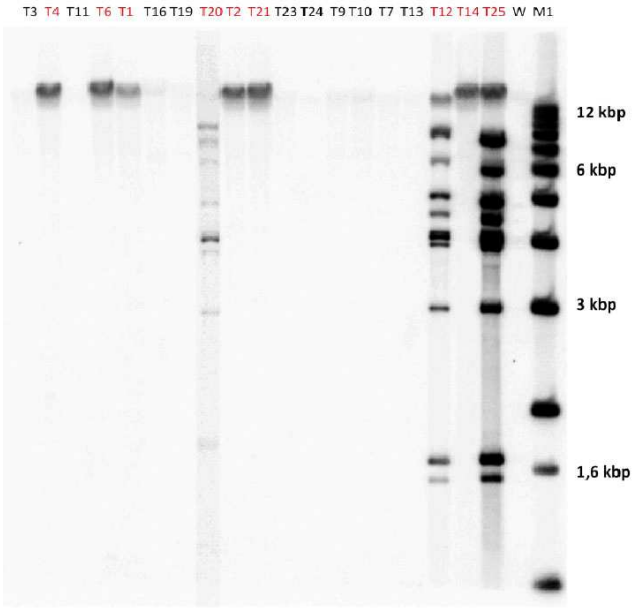


**Fig. 3. Southern blot of transplastomic clones of *C. reinhardtii* with an *aadA* probe.**

Isolated DNA was digested with SacI. 787b fragment of aadA gene prepared Lanes T3 - T25, different potentially transgenic lines; lane W, untransformed *C. reinhardtii;* lane M1, Mw standard 1 kb DNA (Gibco BRL).

Nine clones were positive with a *aadA* probe. Two of them, T4 and T6 with clean, strong signal were selected for Western blot analysis (Fig. 4). E7GGGp/aadA fusion protein could be detected in 10 times concentrated algal extracts only. The band of appropriate size of fusion protein 40 kDa was found accompanied by lower weight degradation products as well as by some higher Mw protein aggregates.

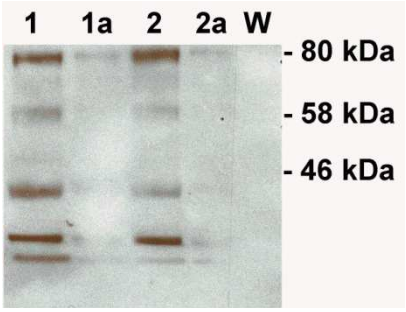


**Fig. 4. Western blot of transplastomic algae extracts expressing *E7GGGp/aadA* cassette.**

300 ml of algal extract was precipitated with ethanol and diluted in 30 ml of loading buffer. Lane 1 and 1a, T4 clone, 20 and 5 ml of concentrated extract applied; lane 2 and 2a, T6 clone; lane W, untransformed algal extract.

**Immunogenicity of HPV 16 E7GGGp/fusion protein expressed in *C. reinhardtii* chloroplasts.**

For immunization of mice only T4 transplastomic clone and untransformed algae were used. Crude algal extracts in PBS buffer supplemented with adjuvants enhancing antibody production were injected subcutaneously and antibodies against the HPV16 E7 were assayed in animal sera. Control immunization was performed with E7 peptide 8Q carrying epitope stimulating T-lymphocytes or with PBS buffer. Immunostimulatory CpG motifs stimulating Th1 lymphocytes or Freud´s adjuvant stimulating B-lymphocytes were used as adjuvant. High titres of Anti-E7 antibodies were detected in mice immunized with transplastomic *C. reinhardtii* extracts with insignificant difference between different adjuvants. Untransformed algae extracts and controls were negative (Fig. 5).

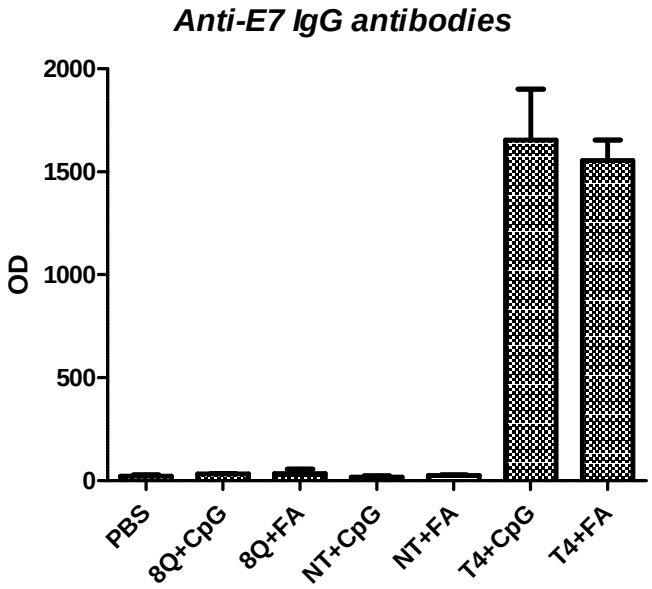


**Fig. 5 Detection of anti-E7 antibodies in mice sera after immunization with untransformed (NT) and T4 transplastomic algal extracts. 8Q E7 peptide and PBS buffer were also used for immunization as controls. CpG: synthetic oligo adjuvant, FA: Freud´s adjuvant.**

Two different classes of induced antibodies were also determined using secondary antibodies. Both IgG1 anti-E7, produced by Th2 helper lymphocytes and IgG2a anti-E7 produced by Th1 helper lymphocytes could be detected in mice injected with transplastomic algae extracts, with insignificant difference in their titer (Fig. 6)

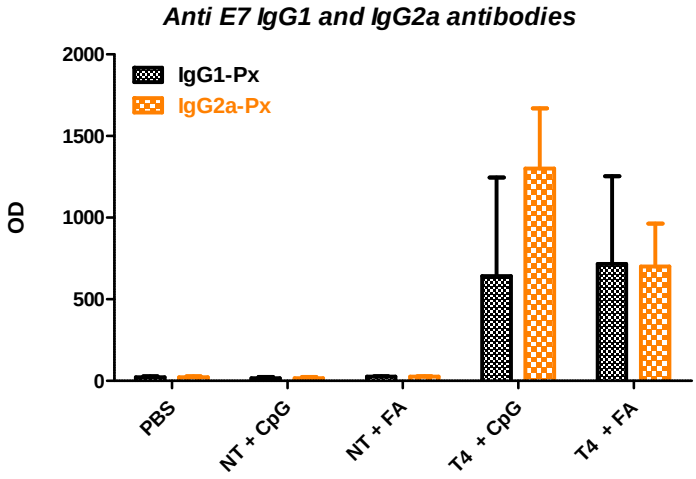


**Fig. 6 Anti-E7 IgG1 and IgG2a antibodies.**

Cellular immunity was assayed using the ELISPOT test. After stimulation of immunized mice lymphocytes with E7-derived stimulating peptide, production of IL-4, indicating Th2, CD4+ helper induction was detected. Nevertheless, IL-4 could be detected also in unstimulated lymphocytes and in mice immunized with untransformed algae, even if to a lesser extent (Fig. 7).

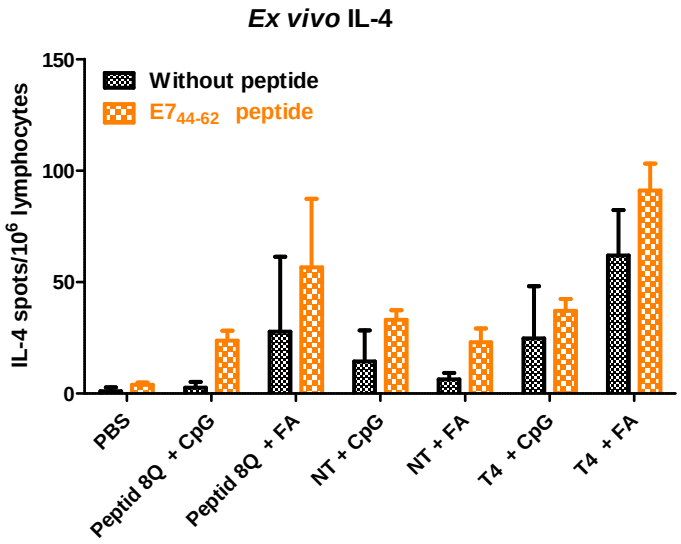


**Fig. 7. Detection of E7-specific CD4+ T lymphocytes by ELISPOT assay. IL-4 -producing cells were detected 1 day after stimulation with the E7 (44-62) peptide. Control lymphocytes were cultivated without peptide.**

IFN-γ indicating Th1, CD8+ activation could be detected only with immunogenic Q8 peptide used as a positive control (Fig. 8).

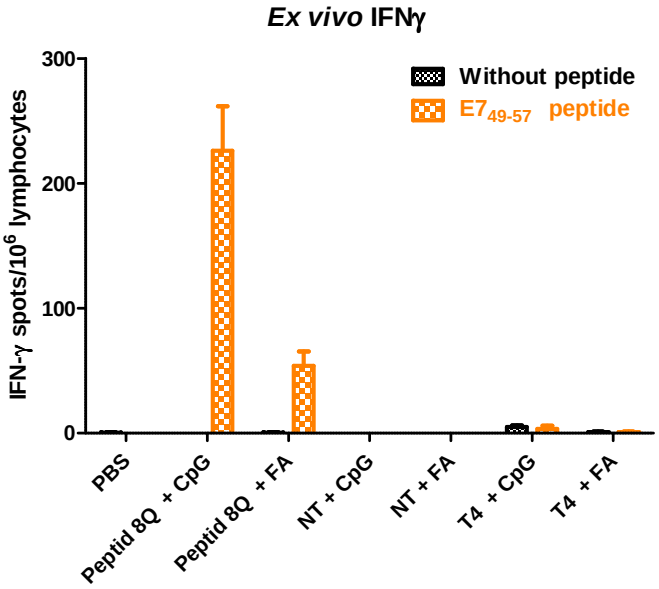


**Fig. 8. Detection of E7-specific CD8+ T lymphocytes by ELISPOT assay. IFNγ -producing cells were detected 1 day after stimulation with the E7 (49-57) peptide. Control lymphocytes were cultivated without peptide.**

## DISCUSSION

We constructed chloroplast vector equipped with long synthetic flanks on both sides of the chloroplast expression cassette which can be advantageous for the convenient production of vector DNA by PCR reaction with flank-derived primers. Other expression cassettes can be also integrated in the vector in the course of PCR reaction, avoiding the time-consuming construction of plasmid intermediates. Such linear vectors are often used in yeast targeted mutagenesis [24] and they were successful also in cyanobacteria transformation [25] that is supposedly controlled by similar mechanisms as in chloroplasts. The testing of efficacy of linear chloroplast vector was one of the aims of the present study but no stable transformants could be obtained. Similar results we have obtained in experiments with tobacco chloroplast transformation [12]. The instability of linear dsDNA in chloroplasts is the most probable cause of linear vector inefficiency. Our results with tobacco also suggest that chloroplast genomes with reduced stability may result from linear vector recombination [12]. Big differences in the expression and stability of *Chlamydomonas* chloroplasts individual transformants were determined [26] and explained by chloroplast genome modification caused by the process of vector recombination.

E7 oncoprotein of the "high-risc" human papillomavirus 16 is an unstable protein with low immunogenicity caused by its quick degradation [6, 22]. We have designed fusion gene of the mutated HPV16 E7 oncogene (E7GGGp) with optimized codon bias and three mutations reducing oncogenicity to increase the stability of E7 and enable simple selection of transformants. Fusion protein was detected in transplastomic algae together with some lower weight degradation products. Probably due to increased stability, we elicited strong production of E7-specific antibodies in immunized mice. On the other hand, CTL response was not induced significantly. This is in agreement with findings [27] that destabilization of the E7 antigen enhanced epitope production and CTL responses.

## CONCLUSION

The findings demonstrate production of a fusion protein expressed from a semisynthetic fusion gene *E7/aadA* in chloroplast-engineered *Chlamydomonas reinhardtii.* Human papillomavirus 16 oncoprotein E7 showed higher stability in fusion with spectinomycin-resistance gene *aadA* and augmented humoral immune response in vaccinated mice. The enhancement of cell-mediated immune response was insignificant.

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## REFERENCES

[1]. H. zur Hausen, *Nat. Rev. Cancer*, **2002**, 2, 342–350.
[2]. K. Ghosal, S. Chakrabarty, A. Nanda, *Der Pharmacia Sinica*, **2011**, 2, 152-168.
[3]. L.A. Koutsky, K.A. Ault, C.M. Wheeler, D.R. Brown, E. Barr, F.B. Alvarez, L.M. Chiacchierini,
[4]. D.M. Harper, E.L. Franco, C. M. Wheeler, A.B. Moscicki, B. Romanowski, C.M. Roteli-Martins,
[5]. I.H. Frazer, *Nat. Rev. Immunol.*, **2004**, 4, 46–54.
[6]. M. Šmahel, I. Poláková, D. Pokorná, V. Ludvíková, M. Dušková, J. Vlasák, *Int. J. Oncol.*, **2008**, 33, 93-102.
[7]. J.B. Ulmer, U. Valley, R. Rappuoli, *Nat. Biotechnol.*, **2006**, 24, 1377–1383.
[8]. I.A.J. Dreesen, G. Charpin-El Hamri, M. Fussenegger, *J. Biotechnol.*, **2010**, 145, 273–280.
[9]. E.L. Giudice, J.D. Campbell, *Adv. Drug Deliv. Rev.*, **2006**, 58, 68–89.
[10]. J. Sambrook, E.F. Fritsch, T. Maniatis; Molecular Cloning: A Laboratory Manual. Cold Spring Harbor, **1989**.
[11]. H.-U. Koop, K. Steinmuller, H. Wagner, Ch. Rosslerh, Ch. Eibl, L. Sacher, *Planta*, **1996**, 199, 193–210.
[12]. J. Bříza, J. Vlasák, S. Ryba, V. Ludvíková, H. Niedermeierová, *Biotechnology & Biotechnological Equipment* , **2013**, *in press*
[13]. U.K. Laemmli UK, *Nature*, **1970**, 227, 680-685.
[14]. M. Bakshi, F. Ebrahimi, A. Hajizadeh, H.K. Alikhani, *Eur. J. Exp. Biol.* **2012**, 2, 1672–1679.
[15]. D.S. Gorman, R.P. Levine, *Proc. Natl. Acad. Sci. USA*, **1965**, 54, 1665–1669.
[16] C.G. Unakal, V.S. Goudar, A. Naregal, H.V. Pavan *et al.*, *Eur. J. Exp. Biol.* **2012**, 2, 1087–1094.
[17]. M. Goldschmidt-Clermont, *Nucleic Acids Res.*, **1991**, 19, 4083–4089.
[18]. P.T. Pratheesh, G.M. Shonima, J. Thomas, C.I. Abraham, K.G. Muraleedhara. *Adv. Appl. Sci. Res.* **2012**, 3, 2679–2686.
[19]. G.M. Church, W. Gilbert, *Proc. Natl. Acad. Sci. USA*, **1984**, 81, 1991– 1995.
[20]. P. Sehr, K. Zumbach, M. Pawlita, *J. Immunol. Methods*, **2001**. 253, 153–62.
[21]. P. Sehr, M. Muller, R. Hopfl, A. Widschwendter, M. Pawlita, *Journal of Virological Methods*, **2002**, 106, 61–70.
[22]. M. Šmahel, D. Pokorná, J. Macková, J. Vlasák, *J. Gene Med.*, **2004**, 6, 1092-1101.
[23]. N.H. Loc, N.S. Cong, D.V. Giap, N.T.Q. Hoa, H.T. Quang, N.D. Huy, *Eur. J. Exp. Biol.* **2012**, 2, 913-918.
[24]. R.J. Rothstein, *Methods Enzymol.*, **1983**, 101, 202-211.
[25]. D. Dauvillee, L. Hilbig, S. Preiss, U. Johanningmeier, *Photosynthesis Res.*, **2004**, 79, 219–224.
[26]. R. Surzycki et al., *Biologicals*, **2009**, 37, 133–138.
[27] W.J. Liu, K.N. Zhao, F.G. Gao, G.R. Leggatt, G.J. Fernando, I.H. Frazer, *Vaccine*, **2001**, 20, 862–869.