A Chlamydomonas-Derived Human Papillomavirus 16 E7 Vaccine Induces Specific Tumor Protection

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Abstract

Background: The E7 protein of the Human Papillomavirus (HPV) type 16, being involved in malignant cellular transformation, represents a key antigen for developing therapeutic vaccines against HPV-related lesions and cancers. Recombinant production of this vaccine antigen in an active form and in compliance with good manufacturing practices (GMP) plays a crucial role for developing effective vaccines. E7-based therapeutic vaccines produced in plants have been shown to be active in tumor regression and protection in pre-clinical models. However, some drawbacks of in whole-plant vaccine production encouraged us to explore the production of the E7-based therapeutic vaccine in Chlamydomonas reinhardtii, an organism easy to grow and transform and fully amenable to GMP guidelines.

Methodology/Principal Findings: An expression cassette encoding E7GGG, a mutated, attenuated form of the E7 oncoprotein, alone or as a fusion with affinity tags (His6 or FLAG), under the control of the C. reinhardtii chloroplast psbD 5′ UTR and the psbA 3′ UTR, was introduced into the C. reinhardtii chloroplast genome by homologous recombination. The protein was mostly soluble and reached 0.12% of total soluble proteins. Affinity purification was optimized and performed for both tagged forms. Induction of specific anti-E7 IgGs and E7-specific T-cell proliferation were detected in C57BL/6 mice vaccinated with total Chlamydomonas extract and with affinity-purified protein. High levels of tumor protection were achieved after challenge with a tumor cell line expressing the E7 protein.

Conclusions: The C. reinhardtii chloroplast is a suitable expression system for the production of the E7GGG protein, in a soluble, immunogen form. The production in contained and sterile conditions highlights the potential of microalgae as alternative platforms for the production of vaccines for human uses.

Introduction

Plant molecular pharming represents a well-established biotechnology area that includes the production of protein biopharmaceuticals such as enzymes, hormones, antibodies, and vaccine antigens in plant systems. Plant-produced proteins represent a significant fraction of pharmaceuticals in advanced preclinical and clinical trial status [1,2]. However, plant platforms present some drawbacks, including long time to generating stable transgenic lines, non homogeneous protein production in different tissues, impact of pests and diseases even in controlled conditions (greenhouses) and, more importantly, growth in non-sterile conditions that make difficult the application of good manufacturing practices (GMP) necessary for the production of pharmaceuticals. To circumvent some of these drawbacks, transient expression [3] or in vitro culture [4] have emerged as alternative platforms. FDA has recently approved the first plant-made drug for human use, an enzyme produced in genetically engineered carrot cells for treating type 1 Gaucher’s disease [5].

Microalgae have been proposed as an alternative molecular pharming system. This relatively new platform offers several advantages, including: 1) short time from transformation to scaling up; 2) rapid growth (doubling time of few hours) and ease of cultivation; 3) safety, because microalgae do not harbor human pathogens, many are Generally Regarded As Safe (GRAS) organisms, and grow in axenic conditions facilitating production of biopharmaceuticals in GMP conditions; 4) homogeneity of protein production with the use of controlled bio-reactors. In particular, the green unicellular alga Chlamydomonas reinhardtii has emerged as a model system, with its three genomes (nuclear, plastidial and mitochondrial) completely sequenced [6], and the easy generation of stable transgenic or transplastomic lines in few weeks [7,8]. While expression from the nuclear genome is subject to position effects [9] and gene silencing [10], expression from the chloroplast genome is well established [11,12]. Like bacteria, the chloroplast lacks the machinery to perform complex post-translational modifications such as glycosylation (the glycosylated proteins come from the Endoplasmic Reticulum), but, unlike E.
HPV Therapeutic Vaccines in Microalgae

**Results and Discussion**

Transformation of the *Chlamydomonas* chloroplast with a mutated form (E7GGG) of the HPV16 E7 oncoprotein

To avoid safety concerns associated with the administration of an oncoprotein in humans, we expressed an attenuated (mutated) form of the E7 protein, named E7GGG, lacking the pRb interaction ability that causes oncogenic transformation of mammalian cells [31]. We expressed E7GGG itself and two affinity tag fusions, E7GGG-FLAG [32] and E7GGG-His6 [33]. The corresponding coding sequences were codon-optimized for chloroplast expression [34] and placed under the control of the psbD promoter and 5’UTR and psbD terminator and 3’UTR (expression cassette) (Figure S1). The chloroplast transformation vector pCG2 (Figure 1A) was obtained by insertion of the expression cassette in the pCG1 vector, which also contained a spectinomycin-resistance cassette and two fragments of the *psaA* intron (5’ and 3’ flanking in Figure 1A) that mediate insertion in the chloroplast genome by homologous recombination.

All constructs were introduced in the cell wall-less (*cw15*) mutant strain of *C. reinhardtii* by transformation with glass beads [35]. Chloroplast transformants were selected on TAPagar plates containing 100 µg/ml spectinomycin and screened by PCR for integration in the chloroplast genome (Figure S2). After at least 10 rounds of streaking for single colonies in selective medium, transformants were also screened for homoplasmy. For all three constructs, transgenes resulted stably integrated in the chloroplast genome and homoplastic cell lines were obtained (Figure S2).

Production and purification of soluble E7GGG protein variants

For each construct (Figure 1A), 40 independent transformants were brought to homoplasmy and screened through Western blotting. All three protein variants were expressed, at different levels, in 85–95% of the analyzed transformants. The four best producers for each E7GGG variant were compared to evaluate possible influences of the affinity tag on maximum accumulation of the E7GGG protein. Higher levels were obtained for E7GGG and E7GGG-FLAG than for E7GGG-His6 (Figure 1B). An inhibitory effect of the His6 tag on chloroplast expression has been observed by others (S. Mayfield, personal communication). This inhibitory effect may be due to impairment of a chloroplast function by the His6 peptide, as suggested by the fact that the best E7GGG-His6 expressor shows almost 50% inhibition of cell growth (Figure S3). In spite of this inhibition, we were able to obtain measurable levels of soluble E7GGG-His6 in *Chlamydomonas* chloroplasts, while we have been unsuccessful in obtaining the same protein variant by Potato Virus X (PVX)-mediated transient expression in *Nicotiana benthamiana* leaves (data not shown).

We compared the amounts of E7 and E7GGG proteins produced in *N. benthamiana* by PVX-infection [27] to those of E7GGG from *C. reinhardtii*. Both forms produced in *N. benthamiana* were present at lower concentrations with respect to the E7GGG protein produced in *Chlamydomonas* (Figure 2). Additionally, the attenuated E7GGG form produced in *N. benthamiana* shows some instability in the absence of protase inhibitors. We also tested the extractability, using different buffers, of E7GGG produced in *N. benthamiana* and in the *Chlamydomonas* chloroplast. In all buffers, the plant-expressed protein was mostly found in the insoluble fraction, while the *Chlamydomonas*-expressed one was highly soluble (Figure S4).

All three protein variants expressed in *Chlamydomonas* accumulated almost exclusively in the soluble cellular fraction (Figure 3A). Protein quantification was performed by immunoblotting, using...
different amounts of E7GGG-His6 protein purified from E. coli as a standard (Figure 3B). The maximum protein yields were about 0.02% TSP for E7GGG-His6, 0.1% for E7GGG and 0.12% for E7GGG-FLAG. These yields are coherent with results obtained with other proteins expressed in the Chlamydomonas chloroplast [8].

A similar yield (0.1% TSP) was obtained for the E7 protein in transplastomic tobacco plants [28].

Up to now, purification of the E7 or E7GGG proteins from plants has been described only for fusion forms with carrier polypeptides such as bacterial lichenase [26,36], or HPV L1 and E6 proteins [37]. We purified the E7GGG-FLAG protein by affinity chromatography on anti-FLAG M2 affinity resin (see Materials and Methods). Good protein recovery was obtained when using 1M Arg-HCl pH 3.5 as elution buffer (Figure S5) [38]. The eluted protein is detectable using the Oriole fluorescent stain (Bio-Rad) (Figure 4). After dialysis against PBS 1X + 0.1 mM ZnSO4 and concentration, about 70% of the original protein was recovered with a final yield of about 7 mg of purified E7GGG-FLAG protein/liter of Chlamydomonas culture.

Purification of the E7GGG-His6 protein was performed using the Ni-NTA resin, with a yield of 1 μg of protein/liter of Chlamydomonas culture. Both in the crude extract or as purified protein, E7GGG-His6 is present as two bands with different apparent molecular weight (MW) of 16 kDa and 18.5 kDa. The two bands were present also after the addition of 10 mM 2-mercaptoethanol and 10 mM dithiothreitol (DTT) and boiling for more than 10’ (Figure S6A). Upon separation on 15% SDS-PAGE, a third band was observed, which increased after calf intestinal phosphatase (CIP) treatment (Figure S6B). This result suggests that the two fastest-migrating bands represent phosphorylated forms of the protein.

Induction of immune responses and protection of mice against E7-expressing tumors by the E7GGG vaccine

Preliminary experiments had already shown that the Chlamydomonas extract (both in the absence or presence of the E7GGG protein) had no toxic effect on mice when injected subcutaneously. Therefore, groups of 8 C57BL/6 mice were immunized 5 times, at 2-week intervals, by subcutaneous administration of the following preparations (in the presence of 10 μg/mouse of QuilA adjuvant): (i) soluble algal extract containing the E7GGG protein (1 mg of TSP containing 1 μg of E7GGG/mouse); (ii) purified E7GGG-FLAG protein from Chlamydomonas (2 μg of protein/mouse); (iii) purified E7GGG-His6 from E. coli (2 μg of protein/mouse). As negative controls, mice were vaccinated with either buffer alone, or with C. reinhardtii.
extract devoid of E7GGG. Both E7GGG-His6 purified from E. coli and E7GGG-FLAG purified from Chlamydomonas induced high titers of specific IgGs after the fourth boost, while the Chlamydomonas E7GGG-containing extract showed a much lower IgG induction (Figure 5). Insignificant IgG induction was detected in the two control groups.

Since humoral immune responses play a marginal role in anti-cancer responses, while the induction of E7-specific cell-mediated (in particular CD8+ T cells) immune response is generally correlated to anti-cancer activity [39], we investigated the presence of the latter by Enzyme-Linked Immunosorbent Spot (ELISPOT) assay. Higher numbers of IFN-γ-secreting cells were detected in mice vaccinated with the E7GGG protein from C. reinhardtii, both in crude extracts and as purified protein, compared to the group vaccinated with E. coli E7GGG-His6 (Figure 6). No, or very few, IFN-γ-secreting cells were detected in the control groups.

Cell-mediated immune responses were also evaluated by measuring the Delayed-Type Hypersensitivity (DTH) response (that represents antigen-specific cytokine mediated inflammation involving Th1 type cytokines) [40] to the HPV16 E7 protein in vaccinated mice, before challenge with the TC-1 cells. An E7-specific DTH response was observed in mice vaccinated with both the Chlamydomonas E7GGG-FLAG antigen and with the Chlamydomonas E7GGG-containing extract (Table 1). In the latter case, the response exceeded that recorded in the group vaccinated with the E. coli E7GGG-His6 (Figure 6). No, or very few, IFN-γ-secreting cells were detected in the control groups.

Tumor protection was evaluated by challenging vaccinated mice with 5×10⁴ cells from the TC-1 tumor cell line, expressing...
the E7 antigen, two weeks after the last injection [26,27,29,41]. Final data collected 13 weeks after the challenge showed that Chlamydomonas crude extract containing E7GGG, as well as purified E7GGG-FLAG from Chlamydomonas and E7GGG-His6 from E. coli elicited tumor protection in 60% of mice (Figure 7). The group of mice vaccinated with the Chlamydomonas E7GGG-FLAG purified protein remained tumor-free for a longer time (100% tumor-free mice after 9 weeks) than the other two groups.

Insignificant protection was observed in the two control groups. Taken together, our data indicate that the microalga-produced E7GGG protein is highly immunogenic, both in crude extracts and in purified form, providing effective tumor protection in a preclinical system.

Conclusions

Since biopharmaceuticals have become increasingly important for the treatment of various human diseases, it is very important to improve existing expression systems as well as to develop new ones. In this context, the benefits arising from molecular pharming and, in particular, from the use of microalgae as production platforms (i.e. ease of scale-up, GMP production, lower production costs), represent a highly attractive perspective.

Important clinical successes have been obtained on patients with HPV16-induced precancerous lesions, treated with vaccines comprising HPV16 E6 and/or E7 proteins [42–44]. Here, we show that an attenuated form of the HPV E7 protein can be

**Table 1.** Delayed type hypersensitivity to E7 protein in vaccinated mice.

| Treatment                                      | Δ ear thickness ± S.D. at 48 h |
|------------------------------------------------|-------------------------------|
| Buffer                                         | 1.0 ± 1.1                     |
| Chlamy wt                                      | 1.8 ± 1.6                     |
| Chlamy E7GGG-containing extract                | 19.3 ± 2.5                    |
| Chlamy E7GGG-FLAG                              | 9.5 ± 2.0                     |
| E. coli E7GGG-His6                             | 13.5 ± 2.6                    |

S.D., standard deviation.

*Ear swelling was reported as the mean of the differences (Δ) in thickness between challenged and unchallenged control ears from five mice per group (mm ear thickening \( \times 10^{-2} \)).

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Figure 6. ELISPOT analysis of splenocytes of vaccinated mice. Splenocytes were recovered from sacrificed animals after the last boost and stimulated with 1 µg/ml of specific CTL E7 peptide (amino acids 49-57, RAHYNVTF) (blue bars) or not stimulated (red bars). The number of IFN-γ producing E7-specific T-cell precursors was determined using an anti-IFN-γ antibody. Data are presented as mean number of spots per 10⁶ splenocytes. Error bars represent standard deviation of three technical replicates.

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produced in the *Chlamydomonas* chloroplast in a highly soluble form, affording cancer protection in a preclinical animal model. Although the amounts of protein produced are still insufficient for clinical experimentation, the present work shows the possibility of using microalgae for the production of bio-active HPV E7 antigen. Additionally, the obtainment of soluble, purified E7 protein from microalgae offers the possibility to perform detailed biochemical and chemical/physical studies, aimed at verifying its structure and biological activity, which up to now have been performed only on proteins expressed in bacterial systems [45,46]. Future developments on gene expression optimization (i.e. the use of different promoters and/or the integration of the transgene in more suitable regions of the chloroplast genome) could lead to increased protein yields, needed for clinical studies.

**Materials and Methods**

**Ethics statement**

Animal experiments performed in this study were conducted according to the Institutional animal use guidelines and the Italian law DL 116/92. All procedures for animal experiments were approved by the Ethical Committee for Animal Experimentation of the Istituti Fisioterapici Ospitalieri (IFO) at the Regina Elena National Cancer Institute. Mice were anesthetized with 40–50 mg of Zoletil (tiletamine+zolazepam Virbac, Milan, Italy) per 1 kg of body weight and all efforts were made to minimize suffering.

**Synthesis of HPV16 E7 gene variants**

We used the mutated form of the HPV16 E7 protein (NP_041326) named E7GGG, firstly realized for genetic vaccination studies by Smahel and colleagues [31]; it presents the mutated GLYGYG amino acid sequence instead to the native DLYC-YE (amino acids 21–26) motif. The E7GGG protein was fused to two purification tags generating two variants: E7GGG-His6 (carrying the hexa-histidine tag and the thrombin cleavage site fused to the N-terminus); and E7GGG-FLAG (carrying the DYKDDDDKs FLAG tag [18], fused to the C-terminus). Codon optimization was performed using the Optimizer application (http://genomes.urv.es/OPTIMIZER) as described [34], aiming at a CAI (“Codon Adaptation Index”) value of 0.8. The optimized nucleotide sequence was synthesized by Genscript Co., USA. Restriction sites NdeI and EcoRI were inserted at the 5’ and 3’, respectively, of the gene, in order to subsequently re-place the gene with the other E7GGG variants, and restriction sites XmaI and NotI were placed at the 5’ and 3’, respectively, of the expression cassette, to introduce it into the chloroplast transformation vector pCG1 (kindly provided by Prof. J. D. Rochaix, University of Geneva). To obtain the E7GGG gene, the E7GGG-His6 sequence was amplified with the forward primer 5’-TTACATCATGACGCGGTGA-TACGCTACATT-3’ (the NdeI site is underlined and the start codon is in bold) and the reverse primer 5’-TTGAATTC-TAGTTAGGTTTTGTGAACAAATAG-3’ (the EcoRI site is underlined and the stop codon is in bold). To obtain the E7GGG-FLAG gene we created a pCG2 vector harboring the FLAG-tag (indicated as pCG2-FLAG) followed by a stop codon upstream the pshA terminator by annealing the following primers and cloning them in the NdeI and EcoRI sites of pCG2: 5’-TTGATTGG-GAAGA1CTGactataaagtagtagtagaataactaTAAGAGC TTAGAgtattaactactactactCtta TTATT-3’ (NdeI, EcoRI and BglII sites are underlined, the FLAG tag in small case, the stop codon in bold and sticky ends are in italic). Subsequently, the E7GGG-His6 sequence was amplified with the same forward primer used to obtain the E7GGG gene, and the reverse primer 5’-ATAAAGA1CTTAGGTTTTGTGAACAAATAGGA-3’ (the BglII site is underlined). The amplicon obtained was then digested with NdeI and BglII and cloned in the pCG2-FLAG vector obtaining the E7GGG-FLAG gene inserted between the pshD promoter/5’UTR and the pshA terminator/3’UTR.

**Chlamydomonas strains, transformation and growth conditions**

For chloroplast transformation, the *C. reinhardtii* cell wall-less strain *ca15* was grown photomixotrophically until mid-log phase (concentration of 5×10⁶ cells/ml, measured using the TC10 automated cell counter, Bio-Rad) in TAP medium at 25°C under constant illumination of 120 μM m⁻² s⁻¹ on a rotary shaker. Cells were harvested by centrifugation and re-suspended in TAP medium to a final concentration of 3×10⁷ cells/ml. Chloroplast transformation was performed as previously described [35]. For each transformation, 300 μl of concentrated cells (about 10⁸ cells/ml) were agitated by vortexing for 20 s in 3,000 rpm in presence of 300 mg of glass beads (acid washed, Sigma, 425–600 μm) and 5 μg of plasmid DNA, and then spread onto TAP/agar plates containing 100 μg/ml spectinomycin. 50 transfectants for each

**Figure 7. Mouse protection against TC-1-induced tumor.** Two weeks after the last boost, five vaccinated mice for each group were challenged by subcutaneous injection of 5×10⁶ TC-1 cells/mouse. The presence of the tumor was monitored by palpation twice a week. Data are represented as percentage of tumor-free mice. doi:10.1371/journal.pone.0061473.g007
construct were propagated for 10 rounds in selective medium to obtain homoplasmic lines. For protein expression and characterization, transformants were grown in the same condition described for the pCEI5 strain with the addition of spectinomycin 100 µg/ml in the culture media. To verify the correct integration in the chloroplast genome and the obtainment of the homoplasmic state, total DNA was extracted from all transformants with a standard phenol/chloroform extraction protocol. Correct integration and homoplasy were checked by amplification with the primers indicated in Figure S2.

Protein extraction and Western blot
For screening of transformants to identify the best expressor lines, 2×10⁶ C. reinhardtii cells were directly re-suspended in a suitable volume of loading buffer (10% glycerol, 60 mM Tris-HCl pH 6.8, 0.025% bromophenol blue, 2% SDS, 3% 2-mercaptoethanol) and boiled for 5′ before loading on a 12% SDS-PAGE gel. To analyze the solubility of the E7GGG protein variants cells were re-suspended in 1/20 culture volume of different buffers (described in Figure S4) and lysed on ice by sonication at 10 Hz output (3×10 seconds). N. benthamiana extracts were prepared by grinding the tissue to a fine powder in liquid nitrogen. The powder was re-suspended and homogenized with an ultraturrax in 3 volumes (w/v) of buffer containing protease inhibitors (“complete, EDTA-free”, Roche Diagnostics, GmbH, Mannheim, Germany). Soluble and insoluble proteins were separated by centrifugation for 20′ at 15,000 g at 4°C, with the resulting supernatant or insoluble pellet used in Western blot analysis. For all other experiments including characterization, purification, mice immunization, soluble proteins extracted using TS buffer (150 mM Tris-HCl, 200 mM sucrose, pH 7.5) were employed. Protein concentration was estimated using the Bradford assay (Bio-Rad Inc., Segrate, Italy). For Western blot analysis, proteins were transferred onto a PVDF membrane (GE Healthcare). After blocking with nonfat milk (5% in PBS), membranes were incubated 2 hours at R.T. with a 1:3,000 dilution of a polyclonal anti-E7 antibody (sera of mice immunized with the purified E7-His6 protein produced in E. coli, kindly provided by Dr. P. Di Bonito, Istituto Superiore di Sanità, Rome). Membranes were then incubated for 1 hour at R.T. with a 1:10,000 dilution of an anti-mouse peroxidase-conjugated secondary antibody (NA931, GE Healthcare) and the bound antibody was detected using the ECL Plus system (“Enhanced Chemi-Luminescence”, GE Healthcare). Protein quantification was performed by lumimetry using a Chemidoc ImageLab system with ImageLab 4.0 software (Bio-Rad).

E7GGG protein purification
Affinity purification of tagged, soluble E7GGG variants was performed in native conditions. The purification of the E7GGG-His6 protein was performed using the Ni-NTA affinity resin (Qiagen), while to purify the E7GGG-FLAG protein we used the anti-FLAG M2 Affinity gel (Sigma). In both cases, after optimization of purification conditions in small scale (100 ml of culture), we performed medium scale purifications (2–10 liters of culture), we performed as previously described [27].

Mice immunization, evaluation of immune responses and tumor challenge
Four-week-old female C37BL/6 mice (Charles Rivers, Como, Italy) were used. Mice were maintained under specific pathogen-free conditions at the Experimental Animal Department of the Regina Elena National Cancer Institute (Rome, Italy). Groups of 8 mice were vaccinated subcutaneously on days 0, 14, 28, 42, and 56 with the following preparations (all in PBS+0.1 mM NaN₃O₄): 1) 200 µl of buffer; 2) 500 µl of soluble extract from wt Chlamydomonas; 3) 500 µl of soluble extract from a Chlamydomonas transformant containing about 1 µg of the E7GGG protein; 4) 200 µl with 2 µg of purified E7GGG-FLAG protein; 5) 200 µl with 2 µg of purified E. coli E7GGG-His6 protein. Adjuvant QuilA (10 µg/mouse) was added to all vaccine preparations. One week after the first and the last boost, all animals were subjected to ELISA and spontaneous Delayed-Type Hypersensitivity (DTH) assays, and after the last boost three animals in each group were sacrificed to evaluated cell-mediated immune responses by Enzyme-Linked Immunosorbon Spot (ELISPOT) analysis. All remaining animals were then challenged by sub-cutaneous injection of 5×10⁶ E7-expressing TC-1 tumor cells [41].

Sera of mice collected one week after the last boosts were analyzed by ELISA assay for the presence of E7-specific antibodies. Microtiter plates were coated with 100 ng/well of E7-His6 protein from E. coli diluted in PBS buffer (pH 7.2) or bicarbonate buffer (50 mM NaHCO₃, pH 9.6). Serial dilution of sera in PBS+2% non-fat milk were added to the coated wells, followed by anti-mouse peroxidase-conjugated secondary antibody (NA931, GE Healthcare) diluted 1:10,000. Colorimetric reaction was induced by adding 100 µl/well v/v H₂O₂/ABTS [2’,2’-azino-bis-(3-ethylbenzotiazolin sulphuric acid) (KPL Inc., Gaithersburg, MD-USA). The ELISA antibody titers were calculated as the log₁₀ of the reciprocal antibody dilution that showed an OD₅₄₅ value above the cut-off value, which was defined as the average OD₅₄₅ value of non-immunized sera±3 standard deviations. DTH was performed as previously described [27]. Ear thickness was assessed 48 and 72 h after challenge using a microcaliper and ear swelling was reported as the difference between the challenged and the unchallenged control ear. HPV16 E7-specific T-cell precursors were detected by ELISPOT as previously described [27]. Briefly, single cell suspension of splenocytes (10⁶ cells/well), harvested from three vaccinated mice/group, was added to microtiter wells coated with a rat anti-mouse IFN-γ antibody (5 µg/ml; clone R4-6A2, BD Bioscience PharMingen, San Diego, CA, USA) along with interleukin 2 (50 units/ml; Sigma-Aldrich, St. Louis, Missouri, USA). Samples were incubated with or without 1 µg/ml of E7-specific H-2Db CTL epitope (aa 49-57, RAHYINIIVTF) [47] at 37°C for 24 h. Plates were then incubated with an anti-IFN-γ biotinylated antibody (2 µg/ml; clone XMG1.2, BD Bioscience PharMingen, San Diego, CA, USA) followed by Streptavidin-HRP (2.5 µg/ml, BD Bioscience PharMingen, San Diego, CA, USA). Spots were developed by adding 3,3’-diaminobenzene/peroxidase substrate Sigma Fast (Sigma-Aldrich St. Louis, Missouri, USA) and counted using a dissecting microscope.

Supporting Information

Figure S1 Sequence of the Chlamydomonas cassette expressing the E7GGG protein variants. The sequence of the E7GGG gene is in red, with the start and stop codons in bold.
The E7GGG-His6 gene variant contains the additional sequence highlighted in blue, that comprises the His6-tag and the thrombin site. The E7GGG-FLAG gene variant contains the additional sequence highlighted in green that comprises the FLAG-tag. Restriction sites are underlined.

(TIF)

Figure S2 Verification of transgene integration and confirmation of homoplasy. A. Integration scheme of the pCG2 plasmid. The integration occurs in psaA intron between nucleotides 158321–160126 (acc. NC_005353.1). The 5' flanking region (5' fl) comprises nucleotides 157103–158321; the 3' flanking region (3' fl) comprises nucleotides 160126–162410. Triangles of different colors joined by horizontal lines indicate primers used to verify correct integration and homoplasy, and the relative amplicons. Amplicons 1 and 2 indicate correct integration, amplicon 3 indicates lack of homoplasy, amplicon 4 is a positive control for the presence of chloroplast DNA. B. Sequences of the primers. C. PCR results of four representative transformants (a–d) after 10 rounds of restreaking on selective medium: the presence of amplicons 1 and 2 confirms the correct integration, the absence of amplicon 3 indicates that the lines are homoplasmic for the presence of the transgene.

(TIF)

Figure S3 Growth curves of the best expressors for each protein variant. Cell concentration (cells/ml) was measured at 0, 12, 24 and 48 hours. Control = transformant obtained with the pCG1 vector. Error bars represent standard deviation of three biological replicates.

(TIF)

Figure S4 Comparison of the solubility of the E7GGG protein produced in C. reinhardtii by chloroplast transformation or in N. benthamiana plants by PVX-mediated infection. Immunoblotting of soluble fraction (S) and insoluble pellet (I) (5 μl each = 20 μg of total proteins in the soluble fraction) from Chlamydomonas and N. benthamiana extracted using the following buffers: (1) 100 mM HEPES-KOH pH 5.0, 200 mM sucrose; (2) 100 mM HEPES-KOH pH 6.0, 200 mM sucrose; (3) 100 mM Tris-HCl pH 7.0, 200 mM sucrose; (4) 100 mM Tris-HCl pH 8.0, 200 mM sucrose; 5) 100 mM Tris-HCl pH 7.0, 154 mM NaCl; (6) 100 mM Tris-HCl pH 7.0, 200 mM sucrose, 1 mM Triton X-100; (7) PBS (21 mM Na₂HPO₄, 2.1 mM NaH₂PO₄, 150 mM NaCl, pH 7.2).

(TIF)

Figure S5 Optimization of E7GGG-FLAG affinity purification. Western of 10 μl of the following samples: lane 1: E7GGG-FLAG extract before purification; lane 2: flow-through; lanes 3, 4: elution with 0.1 M glycine pH 3.5; lanes 5, 6: elution with 0.1 M glycine pH 2.5; lanes 7, 8: elution with 1 M Arg-HCl pH 3.5; lanes 9, 10: elution with 100 μg/ml FLAG peptide; lane 11: empty resin (negative control), e = eluted fraction r = resin after protein elution.

(TIF)

Figure S6 E7GGG-His6 protein characterization. A. Western blot of 20 μg of TSP extracted from the E7GGG-His6 transformant and treated as follows: lane 1: boiling for 5’ in presence of 10 mM 2-mercaptoethanol; lane 2: boiling for 10’ in presence of 10 mM 2-mercaptoethanol; lane 3: boiling for 5’ in presence of 10 mM 2-mercaptoethanol and 10 mM DTT; lane 4: boiling for 10’ in presence of 10 mM 2-mercaptoethanol and 10 mM DTT; lanes 5, 6: purified E7GGG-His6 protein from E. coli 2 and 5 ng, respectively. B. Western blot after calf intestinal phosphatase (CIP) treatment of 20 μg TSP at 37°C. Lane 1: 40 U CIP 30’; lane 2: 40 U CIP 60’; lane 3: untreated 30’; lane 4: untreated 60’.

(TIF)

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Author Contributions

Read and approved the final manuscript: OCD SM PF AV RF GG. Conceived and designed the experiments: OCD GG RF AV. Performed the experiments: OCD GG RF AV. Performed the experiments: OCD SM PF AV. Analyzed the data: OCG GD AV RF. Contributed reagents/materials/analysis tools: GG AV PF RF. Wrote the paper: OCD GG RF.

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