

Expression of Multiple *Taenia Solium* Immunogens in Plant Cells Through a Ribosomal Skip Mechanism

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Abstract *Taenia solium* cysticercosis is a major parasitic disease that affects the human health and the economy in underdeveloped countries. Porcine cysticercosis, an obligatory stage in the parasite life cycle, is a suitable target for vaccination. While several recombinant and synthetic antigens proved to be effective as vaccines, the cost and logistic difficulties have prevented their massive use. Taking this into account, a novel strategy for developing a multi-epitope low-cost vaccine is herein explored. The S3Pvac vaccine components (KETc1, KETc12, KETc7, and GK1 [KETc7]) and the protective HP6/TSOL18 antigen were expressed in a Helios2A polyprotein system, based on the ‘ribosomal skip’ mechanism mediated by the 2A sequence (LLNFDLLKLAGDVESNPG-P) derived from the *Foot-and-mouth disease virus*, which induces self-cleavage events at a translational level. This protein arrangement was expressed in transgenic tobacco cells. The inserted sequence and its transcript were detected in several Helios2A lines, with some lines showing recombinant

protein accumulation levels up to 1.3 µg/g of fresh weight in leaf tissues. The plant-derived Helios2A vaccine was recognized by antibodies in the cerebral spinal fluid from neurocysticercosis patients and elicited specific antibodies in BALB/c immunized mice. These evidences point to the Helios2A polyprotein as a promising system for expressing multiple antigens of interest for vaccination and diagnosis in one single construction.

Keywords Low-cost expression platform · Multicomponent vaccine · Plant-derived immunogen

Introduction

Taenia solium cysticercosis affects both humans and pigs. The larval stage of the parasite establishes in the host tissues after hatching from eggs released by a human tapeworm carrier. Humans, the definitive hosts, acquire the intestinal tapeworm by eating insufficiently cooked meat from cysticercus-infected pigs [1, 2]. A single tapeworm produces tens of thousands of eggs, which are shed to the environment and contaminate vegetables, running waters, and soils when a tapeworm carrier defecates in the open air [2].

T. solium parasitosis is deeply rooted in non-developed countries of Latin America, Africa, and Asia, where low socioeconomic and hygienic standards promote its transmission: rustic pig rearing, inadequate and insufficient meat inspection, improper drainage, and limited access to sanitary services [2]. Neurocysticercosis (NC) is the most frequent clinical manifestation of *T. solium* infection in humans [3]. The role of pigs as obligatory intermediate hosts could enable us to interfere with the transmission cycle by preventing porcine cysticercosis [1]. In this

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Table 1 Peptidic components of Helios2A protein with estimated molecular weight

Component	Molecular Weight
KETc1	≈ 1.2 KDa
KETc12	≈ 0.8 KDa
KETc7	≈ 10 KDa
TSOL18	≈ 18 KDa
GK1	≈ 2.0 KDa
2A	≈ 2.0 KDa

respect, it has been demonstrated that cysticerci are vulnerable to vaccine-induced immunity [4]. Indeed, effective synthetic and recombinant injectable vaccines against porcine cysticercosis have been developed [5–8]. S3Pvac and the HP6/TSOL18 antigen have been thoroughly studied and field-tested for vaccine efficacy [6–8].

The S3Pvac multi-epitope vaccine includes three peptide components expressed in the different developmental stages of *T. solium*. S3Pvac, formulated from either synthetic or recombinant phage-expressed peptides and subcutaneously administered, reduced muscle cysticercosis by 50–54 % and the number of established cysticerci by 87–97 % in pigs naturally exposed to the infection on the field [7]. Since the peptides in S3Pvac are shared by other cestodes [9], the vaccine could also be effective in reducing porcine hydatidosis [10] as a side beneficial effect. The S3Pvac-phage anti-cysticercosis vaccine is now being employed in the State of Guerrero, Mexico, in a control program ongoing since 2009 [11].

HP6/TSOL18, first detected in *T. ovis* and *T. saginata* [12, 13], was later identified in *T. solium* by To18 cDNA hybridization [14, 15]. Once recombinantly expressed in *Escherichia coli*, this 130-amino acid protein proved to induce high protection levels against experimental porcine cysticercosis when subcutaneously administered in independent experimental vaccine trials carried out in Mexico and Cameroon [5].

Both S3Pvac and HP6/TSOL18 are formulated with purified antigens derived from *E. coli* cultures, having technical and economic difficulties that limit their use in developing nations. Plant cells, on the other hand, are increasingly recognized as a valuable platform for vaccine development, offering low costs, absence of mammalian pathogens, and complex antigen biosynthesis. Plant-based antigens offer several economic advantages as well. These systems allow for antigen production at low costs, avoiding the costly step of antigen purification and expensive technologies for artificial antigen encapsulation. They are very accessible and could be an attractive approach for mass immunization in poor countries [16]. The interest of pharmaceutical companies in this type of vaccines and the

number of products close to be marketed are good evidences of the viability and maturity of this technology [17]. Currently, a plant-made vaccine against Newcastle virus has been approved for use by the Center of Veterinary Biologics of the United States Department of Agriculture [18]. While not a vaccine, Protalix BioTherapeutics is marketing a plant-derived product named ELELYSO, a recombinant hydrolytic lysosomal glucocerebrosidase-specific enzyme indicated for long-term enzyme replacement therapy (ERT) for adults with confirmed diagnosis of Type 1 Gaucher disease. This case illustrates the high potential of plant-derived biopharmaceuticals to be marketed in the near future [19].

Considering the above stated advantages, a vaccine against cysticercosis was developed in embryogenic transgenic papaya. S3Pvac-papaya induces high protection levels against *T. crassiceps* and *T. pisiformis* cysticercosis when parenterally or orally administered [20, 21]. While this vaccine has proved efficacious, further improvements are envisioned in light of the molecular tools of plant biotechnology currently available [16, 22, 23]. For example, a simultaneous expression of the vaccine components at higher levels would allow easier formulation and dosage processes. One important innovation in this regard is the use of the 2A peptide from *Picornavirus*, specifically the *Foot-and-mouth disease virus* (FMDV), which mediates the expression of more than one protein through a polyprotein encoded by a single gene [24, 25]. This 20-aa sequence induce self-cleavage events at a translational level by modifying the activity of the ribosome to allow hydrolysis of the ester linkage 2A-tRNA_{gly} to be released, while translation of the downstream product continues. This mechanism is referred to as “ribosomal skip”. The 2A region of the FMDV genome encodes a sequence that mediates self-processing by a novel translational effect, variously referred to as “ribosome skipping” [26], “stop-go” [27], and “stop carry-on” translation [28]. 2A-mediated cleavage occurs into the sequence LLNFDLLKLAGDVESNPG-P between the glycine and the proline residues at the C-terminus of the 2A protein [29, 30]. Briefly, the nascent 2A peptide interacts with the exit pore of the ribosome in such a way that the C-terminal portion (-ESNPGP-) is sterically restricted within the peptidyl transferase center of the ribosome. This inhibits the nucleophilic attack of the ester linkage between 2A and tRNA_{gly} by prolyl-tRNA in the A site—effectively stalling, or pausing, translation [26, 31]. It has been shown that this block is relieved by the action of the translation release factors eRF1 and eRF3, hydrolyzing the ester linkage and releasing the nascent protein [28, 32]. For in-depth reviews of the model, see [33–35].

The 2A peptide sequence is functional in a variety of eukaryotic systems, which include mammalian (human

among them), insect, and plant cells [36]. The design and effective expression of a gene encoding for a polyprotein comprising several peptide components of an anti-*T. solium* cysticercosis is herein described.

Materials and Methods

Gene Design and Vector Construction

A synthetic gene named Helios2A was devised to include the following antigens from *T. solium*: KETc7, GK1, KETc1, and KETc12 [37–39], all of them included in the S3Pvac vaccine, and in addition the TSOL18 antigen. These antigenic sequences were arranged in a single ORF, including the 2A sequence (LLNFDLLKLAGDVESNPG-P) between each sequence of interest. The Helios2A gene (999 bp) was optimized and synthesized by Genscript, including flanking restriction sites (5'*Sma*I, 3'*Sac*I) and inserted into the vector pUC57. Subcloning by digestion with *Sma*I and *Sac*I was performed to insert the gene into the pBI121 vector, substituting the *uidA* reporter gene. A positive clone, selected by restriction analysis, was used for plasmid isolation and then mobilized into the *Agrobacterium tumefaciens* GV3101 strain by electroporation. A recombinant clone was selected for plant transformation experiments. All molecular cloning procedures were performed following standard techniques [40].

Genetic Transformation of Plants

Tobacco (*Nicotiana tabacum* cv. Petite Havana SR1) leaf sections were used for transformation as previously described by Horsch et al. [41]. Transgenic plants were selected on selection/regeneration plates containing 100 mg/l kanamycin for selection of transformants and 250 mg/l cefotaxime for *Agrobacterium* removal. Rooted kanamycin-resistant shoots were transferred to soil and grown in a greenhouse. Plants were grown under a 16-h photoperiod, with a light intensity of 100 $\mu\text{mol m}^{-2}\text{s}^{-1}$, and 30 % relative humidity. Transgenic lines were self-pollinated and seeds were collected. To obtain the T1 generation, seeds were germinated on MS medium containing 100 mg/l kanamycin, and seedlings were then transferred to soil. These transgenic plants were used for subsequent analyses.

Transgene Detection by PCR

Total DNA was isolated from leaves of putative transformants or wild-type (WT) plants according to a previously described protocol [42]. PCR conditions were: A 50 μl reaction mixture containing 100 ng DNA, 1 \times PCR buffer, 1.5 mM magnesium chloride, 2.5 U *Taq* DNA polymerase

(Vivantis), 1 mM dNTPs, and 1 μM of each of forward and reverse primers to amplify the Helios2A gene (Forward, 5'gagcttttatgcaaccacatcc; Reverse, 5'gcggaataaggtggtgcgtag), yielding a 456 bp amplicon. Temperature cycling conditions were: 94 °C for 2 min (initial denaturation); 35 cycles of 95 °C for 30 s (denaturation); 56 °C for 30 s (annealing); 72 °C for 1 min (elongation); and a final extension at 72 °C for 5 min. This procedure was performed in a MultiGeneTM Mini Personal Thermal Cycler (Labnet). PCR products were analyzed by electrophoresis in 1 % agarose gels.

Transcript Detection by RT-PCR

Total RNA was extracted from leaves of transgenic or WT tobacco plants using the Trizol reagent (Invitrogen, USA) according to the manufacturer's instructions. Five hundred nanograms of total RNA were used to generate cDNA with a Taqman reverse transcription kit (Applied Biosystems, Carlsbad, CA). PCR analysis was performed using the primers and amplification protocol described above, to detect a 456-bp amplicon representing Helios2A transcripts. A region of the nitrate reductase gene was amplified as a loading control in parallel reactions, using the following primers: 5'aactgggatgacatggagaa and 5'atcacactcatgatggattgta.

Detection of Plant-Derived Antigens by Western Blot

Total soluble proteins were extracted from freeze-dried leaf tissues, processed in a Labconco chamber overnight (FreeZone[®] 2.5-Liter Benchtop Freeze Dry System). Ten milligrams of leaf powder were resuspended into 50 μl of 1X reducing NUPAGE LDS sample buffer and NUPAGE antioxidant (Invitrogen). Samples were denatured by boiling for 5 min at 95 °C; debris were removed by centrifugation at 12,000 $\times g$ for 10 min. Supernatant was analyzed in a NUPAGE 4–12 % Bis–Tris gel (Novex, Invitrogen). The gel was blotted onto a BioTrace PVDF membrane (Pall Corporation, NY). Protein transfer was performed in TV100-EBK Electrobloater (AlphaMetrix Biotech, GER) for 1 h at 150 V in a methanol-based transfer buffer. After blocking in PBST-0.05 % plus 5 % fat-free dry milk for 5 h at 25 °C, strips were incubated overnight at 4 °C with polyclonal rabbit anti-TSOL18 or anti-GK1 antibody (1:800); *E. coli*-expressed TSOL18 or a poly-GK1 protein, comprising six GK1 tandem repeats in a single polypeptide, were used as positive controls. Binding antibodies were then developed using a horseradish peroxidase-conjugated goat anti-rabbit antibody (1:2000; Sigma, USA) and incubated for 2 h at room temperature. Detection was carried out by incubation with an ECL Western blotting substrate (Thermo Scientific, USA), following the

manufacturer's instructions. Signal detection was verified using an X-ray film exposed for 20 and 40 min, following standard procedures.

Detection of Plant-Derived Antigens by ELISA

About 10 mg of fresh leaf tissues were milled and resuspended in 100 μ l of protein extraction buffer (100 mM NaH_2PO_4 , 8 M Urea, and 0.5 M NaCl; pH 8). Samples were centrifuged at 14,000 rpm in a microcentrifuge for 10 min at 4 °C. Assay plates were coated overnight at 4 °C with protein extracts diluted in carbonate buffer. A standard curve made with pure TSOL18 was traced in a range of 0.5–15 ng/ μ l. Plates were washed with PBST and blocked with 5 % fat-free dry milk for 1 h at room temperature. After washing with PBST, anti-TSOL18 antiserum (1:800) was added and the plates were incubated overnight at 4 °C. A horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (1:2000; Sigma, Missouri, USA) was added and incubated for 2 h. After washing with PBST, a substrate solution composed of 0.3 mg/l 2-20-Azino-bis-3 etilbenztiasoline-6-sulphuric acid (ABTS; Sigma, Missouri, USA) and 0.1 M H_2O_2 was added. Optical density (OD) at 405 nm was recorded in a Multiskan Ascent (Thermo Scientific, Massachusetts, USA) microplate reader.

Detection of Anti-cysticercal Antibodies in CSF

Cerebral spinal fluid (CSF) samples from 23 NC patients attending at the Instituto Nacional de Neurología y Neurocirugía, Mexico City, were kindly provided by Dr. Agnes Fleury. Patients were diagnosed based on radiological studies (CT/NMR). CSF samples were collected from patients who required lumbar puncture for diagnosis and/or follow-up. Seven CSF samples from neurological patients affected by other diseases were used as controls. Patients were informed that CSF samples obtained during their hospital studies would be used for this work and gave their informed consent.

Anti-cysticercal antibodies were detected in CSF samples by ELISA, using soluble proteins from plant cells expressing Helios2A as antigen source, following the procedure previously described [43]. Samples were run in duplicate. A test was regarded as positive when the mean OD reading was higher than cut-off value; the latter was set based on the mean OD plus 2 SD of 10 CSF samples from non-NC controls.

Immunogenicity Evaluation in Mice

All animals were handled in accordance with the Mexican federal regulations for animal experimentation and care

(NOM-062-ZOO-1999, Ministry of Agriculture, Mexico); the experimental protocol was approved by the Institutional Animal Care and Use Committee.

Groups of five 12- to 14-week-old BALB/c female mice, average weight 25 g, were used. Mice were subcutaneously immunized with 10 mg of fresh plant tissue of WT tobacco or the transgenic clone homogenized in 100 μ l of PBS. Mice were immunized once a week for three consecutive weeks, and sacrificed 7 days after the last immunization. Blood was collected and incubated at 25 °C for 1 h to allow clotting, and then centrifuged at 1200 \times g for 5 min at 4 °C to obtain the sera. Anti-cysticercal antibodies were detected by ELISA. Plates were coated with 200 ng of each antigen; 100 μ l of carbonate buffer 0.2 M, pH = 9.6 were added and the plates were incubated overnight at 4 °C. Plates were then washed with PBST-0.05 % and blocked with 5 % fat-free dry milk for 2 h at room temperature. Plates were washed with PBST-0.05 %, and sera from mice immunized either with the Helios2A polyprotein or with WT tobacco were added (dilution 1:10), and incubated overnight at 4 °C. A horseradish peroxidase-conjugated goat anti-mouse IgG (1:2000; Sigma, Missouri, USA) was added and incubated for additional 2 h at room temperature. After washing with PBST-0.05 %, 100 μ l of a substrate solution of 0.3 mg/L 2-20-Azino-bis-3 etilbenztiasoline-6-sulphuric acid (ABTS; Sigma, Missouri, USA) and 0.1 M H_2O_2 was added and incubated for 5 to 10 min at room temperature. OD at 405 nm was recorded in a Multiskan Ascent microplate reader (Thermo Scientific, Massachusetts, USA).

Statistical Analysis

Data were analyzed by one-way ANOVA. Statistical analysis was performed with the Statistica software (version 2.7). Student *t*-tests for independent samples at $P = 0.05$ were also conducted.

Results

Engineered Plants Carry and Transcribe the Helios2A Transgene

Figure 1a shows the sequence of the Helios2A polyprotein. Figure 1b shows the corresponding expression vector based on the pBI121 backbone, successfully constructed as revealed by restriction profiles and sequencing analysis (data not shown).

Following the *Agrobacterium*-mediated transformation procedure, several candidate tobacco lines were isolated in selective media. A total of six putative transformed lines were successfully regenerated and acclimatized in soil.

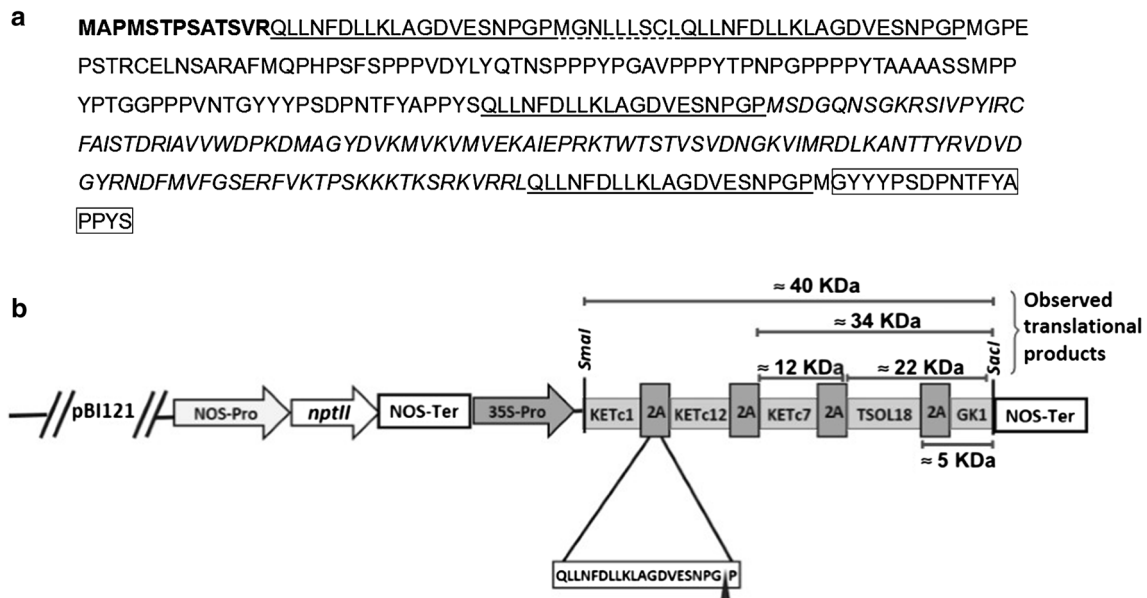


Fig. 1 a Map of the binary vector pBI-Helios2A constructed for the expression of the Helios2A gene in plants. The gene of interest is under control of the native CaMV 35S promoter, along with the 5'UTR from tobacco etch virus. In addition, an *nptII* expression cassette is included to allow for selection of transformed lines. The

expected molecular weight for the distinct Helios2A cleavage products is shown. **b** Amino acid sequence of the Helios2A gene. The sequence is described as follows: KETc1 (*bold letters*), KETc12 (*gray letters*) KETc7, TSOL18 (*italics*), 2A sequence (segmented *underlined letters*), GK1 (*box*)

These putative transgenic lines were designated as T1-2A, T2-2A, T3-2A, T4-2A T5-2A, and T6-2A, and used for further analysis. The presence of the Helios2A transgene was assessed by PCR. The expected amplicons were detected in all analyzed lines, thus confirming presence of the transgene (Fig. 2). These findings confirmed the successful transfer of the Helios2A transgene into tobacco plants.

To ascertain the Helios2A gene transcription, specific transcripts were detected in all six transgenic tobacco lines by RT-PCR (Fig. 3). No major differences in transcript levels were observed among these lines, indicating that the Helios2A transgene was transcribed in each of these lines at similar levels.

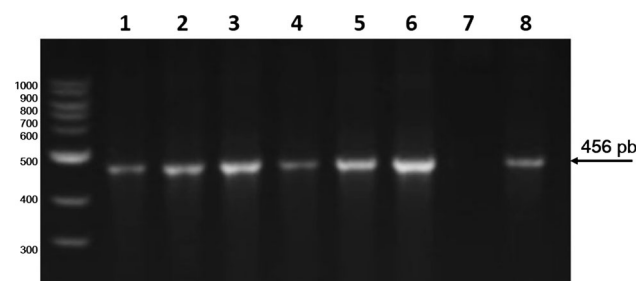


Fig. 2 Detection of Helios2A gene PCR analysis. PCR was conducted using genomic DNA extracted from leaves of transgenic or wild-type tobacco plants and Helios2A-specific primers. Lines: M, 100-bp ladder; 1–6, transgenic lines; 7, wild-type plant; 8, positive control (plasmid pBI-Helios2A)

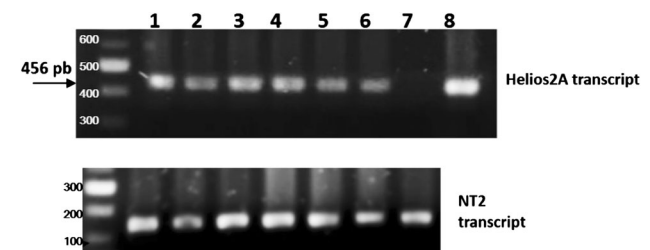


Fig. 3 Detection of Helios2A transcripts by RT-PCR analysis. cDNA from tobacco plants was synthesized and subjected to PCR amplification using primers for Helios2A or a region of the tobacco nitrate reductase (NT2) as housekeeping gene. Lines: M, 100-bp ladder; 1–6, transgenic lines; 7, wild-type plant; 8, plasmid carrying the Helios2A gene as a positive control

Plant-Derived Helios2A Peptides Retained Antigenic Properties

To investigate the efficiency of this 2A-based expression method, a Western blot analysis was conducted. When anti-TSOL18 antibodies were used for labeling, a protein of ≈ 22 kDa that may correspond to TSOL18 and additional epitopes in the Helios2A protein (Fig. 4a) was detected. To better characterize the identity of the recombinant antigens, line T1-2A was selected for assessing detailed expression patterns and antigenicity. When anti-GK1 antibodies were used for labeling, several bands showed positive activity at molecular weight of 5, 12, 22, 34, and 40 kDa (Fig. 4b). The positive control, polyGK1 protein, showed reactivity at 22 KDa, as expected.

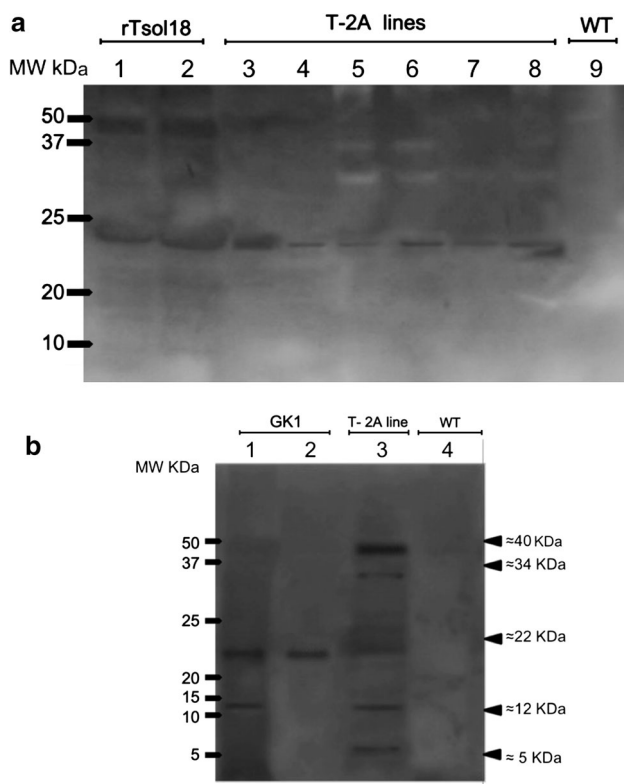


Fig. 4 Immunodetection of *T. solium* antigens in tobacco protein extracts. **a** Western blot was conducted using a rabbit polyclonal anti-TSOL18 antibody. Lines: 1 and 2, pure recombinant TSOL18-GST as positive control (500 and 250 ng, respectively); 3–8, protein extracts from 6 T-2A lines; line 9, protein extract from wild-type tobacco plant. **b** Western blot was conducted using a rabbit polyclonal anti-GK1 antibody. Lines: 1 and 2, pure recombinant tandem repeated GK1 peptide as positive control (100 ng); 3, protein extracts from T-2A line; line 4, protein extract from wild-type tobacco plant

As Fig. 5 shows, the four peptides were detected by ELISA in the soluble extract from the T1-2A line, using polyclonal rabbit antibodies against each peptide. Significantly lower OD values were read with total extract of WT tobacco (data not shown). Similar low OD values were read when pooled sera from non-immunized rabbit were used (Fig. 5a). According to a quantitative ELISA using pure TSOL18 as standard (Fig. 5b), this component accumulated at levels of up to 1.3 μg per gram of fresh leaf tissue.

The reactivity of tobacco-derived antigens with biological fluids from NC patients was assessed by ELISA, using tobacco protein extracts as a target. Figure 6 shows the read OD values when CSF from neurological patients, either with or without NC, were used as antibody source. Significantly higher OD readings were detected in NC-CSF ($P = 0.02$). Detected levels were much higher for T1-2A clone with respect to the WT soluble extract ($P = 0.06$). These findings confirmed the presence of parasite antigens in the T1-2A transgenic line.

Plant-Derived Helios2A Antigens are Immunogenic in Mice

To analyze the immunogenic potential of tobacco tissues expressing the Helios2A gene, mice were subcutaneously immunized either with extract from leaves of T1-2A line adult plants or with WT plant extract. Sera from mice immunized with transgenic tobacco showed significantly higher serum anti-GK1, KETc1, KETc12, and TSOL18 antibody levels with respect to the WT-immunized group ($P < 0.05$) (Fig. 7). These findings demonstrated that Helios2A effectively expressed each of the vaccine peptides, maintaining their immunogenic conformation.

Discussion

The design of an innovative system for the simultaneous expression of five *T. solium* vaccine antigens is herein reported. A polyprotein carrying these antigens was created based on the “ribosomal skip” mechanism mediated by the viral 2A sequence, to release the distinct antigens in a single transformation and expression event.

The coordinated expression of multiple transgenes is crucial for the synthesis of proteins partaking in one same biological effect, such as a metabolic pathway, or when various components are involved in one same objective, like multicomponent vaccines when a number of antigens are administered together to induce broad immunoprotective responses. In animal cells [44–46] and plants [35, 47], the coordinated expression of two ORFs has been achieved using IRES elements (Internal Ribosome Entry Site) or the 2A sequence through a bicistronic system. In nature, both mechanisms are used in viral protein expression systems. IRES, present in viral RNA, relies on the initiation of ribosome binding and translation in a cap-independent manner [48]. On the other side, the 2A viral sequence is found in self-cleaving polyproteins and acts by a “ribosome skipping” mechanism [31, 49]. The latter have been used for biotechnological purposes in rice plants. Both bicistronic systems were used to co-express the genes coding for phytoene synthase (*psy*) and carotene desaturase (*ctrl*), which are required for carotene biosynthesis. Interestingly, the 2A system was better in driving the correct translation of the two proteins [50]. The 2A sequence from the *Foot-and-mouth disease virus* (FMDV) was used in *Chlamydomonas reinhardtii* to co-express the *ble* gene (responsible for resistance to zeocin) and the *xyn* gene, which encodes for xylanase, an enzyme relevant for industrial processes. Co-expression through the bicistronic system led to a 100-fold increase in xylanase production with respect to the monocistronic system [51]. It is noteworthy that a portion of the protein remained unprocessed

Fig. 5 a Antigenic activity of tobacco protein extracts. Wild-type or T1-2A lines were analyzed by ELISA using polyclonal rabbit antibody against each of the vaccine components. Serum from non-immunized rabbits was used as a negative control. Increase in OD values with respect to WT extracts is shown. Asterisks denote significantly higher values with respect to reactivity against unimmunized rabbit sera ($P < 0.01$). **b** Standard curve using pure TSOL18 protein (0.5–15 ng/ μ L) to estimate accumulation levels in fresh leaf tissue

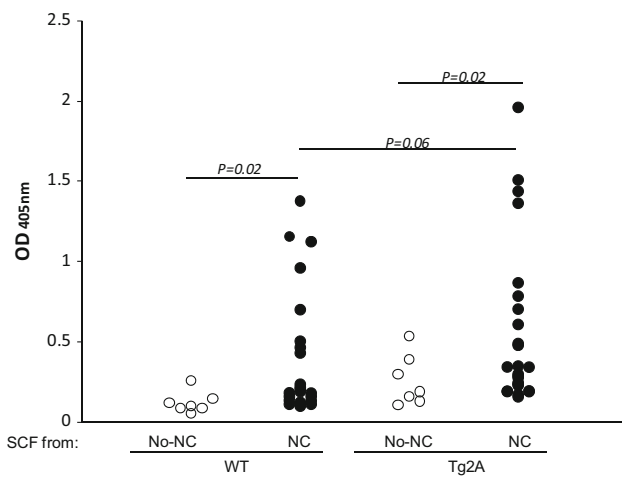
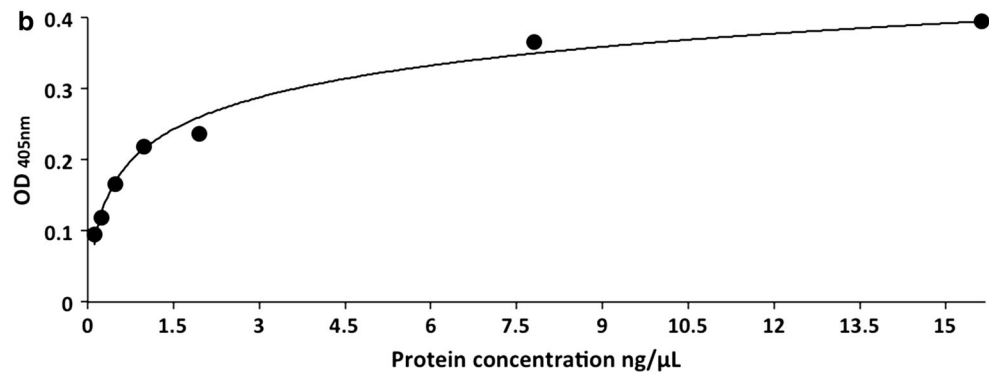
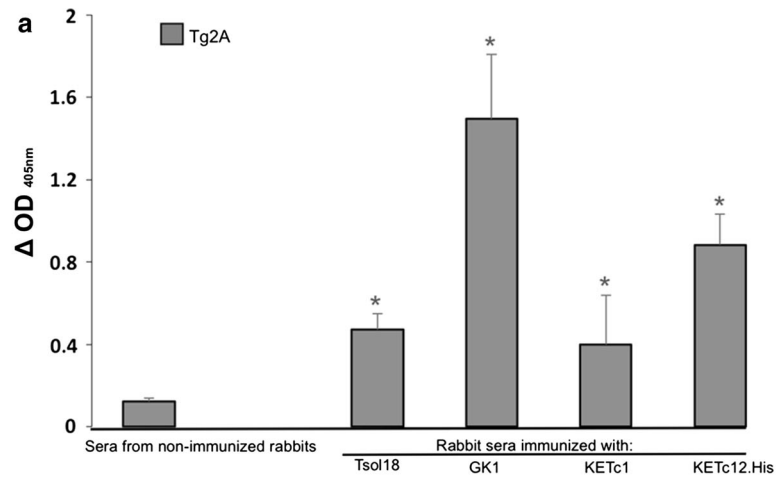


Fig. 6 Detection of anti-cysticercal antibodies in CSF using plant-derived Helios2A antigens. CSF samples from non-NC and NC neurological patients were assayed at 1:10 dilution using soluble extract from wild-type tobacco or T1-2A line for specific antibody detection. OD values are shown and bars denote statistical differences

under this 2A-mediated system in these two reports. In our work, we have simultaneously expressed five different antigens in an arrangement using the 2A sequence from FMDV. In previous efforts to use plants as convenient biofactories for *T. solium* antigens, KETc1, KETc12, and

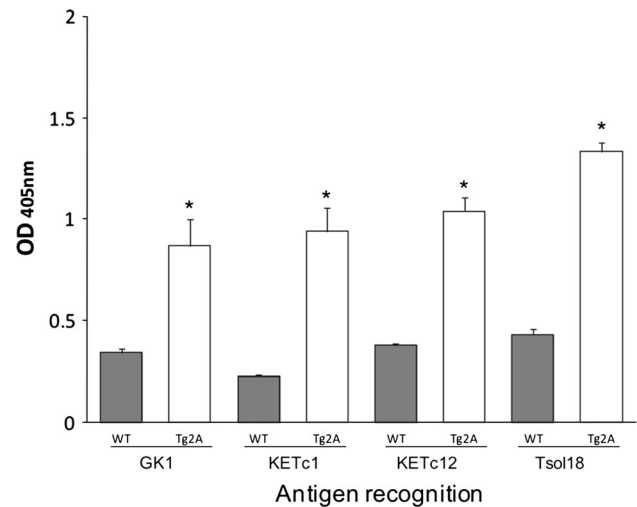


Fig. 7 Levels of antibodies induced by the transgenic tobacco T1-2A line in immunized mice, specific for the GK1, KETc1, KETc12, and TSOL18 antigens (open bars). OD values from mice sera immunized with wild-type tobacco are shown in dark bars. Asterisks denote significantly higher values with respect to WT-treated group ($P < 0.05$)

KETc7 were individually produced in papaya cell lines, leading to a high efficacy vaccine [20]. This study has focused instead on expressing those components, plus the

TSOL18 immunoprotective antigen, in a single clone. This approach will greatly facilitate the formulation and may enhance the protective potential of the vaccine.

Using the 2A expression approach, a ≈ 22 kDa protein was detected when an anti-TSOL18 serum was used. Considering that the expected molecular weight for TSOL18 is 18 kDa, the observed band may correspond to a partial Helios2A cleavage product including TSOL18-GK1 (Fig. 1b). Several bands (5, 12, 22, 34, and 40 kDa) showed positive reactivity to anti-GK1 serum. Taking into account that the GK1 sequence is part of KETc7, a positive reactivity is expected for several Helios2A cleavage products containing GK1 and/or KETc7. Therefore, the 5, 12, 22, and 34 kDa bands are likely due to the following products: GK1 (5 kDa), KETc7 (12 kDa), GK1:TSOL18 (22 kDa), and GK1:TSOL18:KETc7 (34 kDa). In addition, the presence of a 40 kDa protein suggests that a fraction of Helios2A remains unprocessed (Fig. 4a). The reactivity of anti-GK1 serum with the poly-GK1 protein confirms the serum specificity, although a degradation product was also detected.

The co-expression of these antigenic peptides is proposed here as an advantageous system to develop new vaccines and immunodiagnostic procedures. It is remarkable that the peptides expressed in Helios2A are capable to elicit antibodies that specifically recognized the respective peptides, proving that the peptides retained their immunogenic properties. This is an important precedent, as no multicomponent vaccines based on the 2A cleavage sequence had been produced in plants [23]. Moreover, Helios2A antigens were recognized by antibodies against the respective peptides and by sera from NC patients. These findings suggest a potential use of this system in the development of diagnostic tools.

Taken together, these findings suggest that this new system is a valuable tool for new multi-epitope platforms for diagnosis and vaccination. Future studies will determine the immunoprotective potential of this tobacco lines to develop new plant-based multi-epitopic vaccines to prevent porcine cysticercosis, and also to provide new methods for human NC diagnosis.

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References

1. Sciuotto, E., Fragoso, G., Fleury, A., Lacleste, J. P., Sotelo, J., Aluja, A., et al. (2000). *Taenia solium* disease in humans and pigs: An ancient parasitosis disease rooted in developing countries and emerging as a major health problem of global dimensions. *Microbes and Infection*, 2, 1875–1890.
2. De Aluja, A. S. (2008). Cysticercosis in the pig. *Current Topics in Medicinal Chemistry*, 8, 368–374.
3. Sotelo, J. (2011). Clinical manifestations, diagnosis, and treatment of neurocysticercosis. *Current Neurology and Neuroscience Reports*, 11, 529–535.
4. Molinari, J., Rodriguez, D., Tato, P., Soto, R., & Arechavaleta, Solano S. (1997). Field trial for reducing porcine *Taenia solium* cysticercosis in Mexico by systematic vaccination of pigs. *Veterinary Parasitology*, 69, 55–63.
5. Flisser, A., Gauci, C. G., Zoli, A., Martínez-Ocana, J., Garza-Rodríguez, A., Domínguez-Alpizar, J. L., et al. (2004). Induction of protection against porcine cysticercosis by vaccination with recombinant oncosphere antigens. *Infection and Immunity*, 72, 5292–5297.
6. Huerta, M., de Aluja, A. S., Fragoso, G., Toledo, A., Villalobos, N., & Hernández, M. (2001). Synthetic peptide vaccine against *Taenia solium* pig cysticercosis: successful vaccination in a controlled field trial in rural Mexico. *Vaccine*, 20, 262–266.
7. Morales, J., Martínez, J. J., Manoutcharian, K., Hernández, M., & Fleury, G. (2008). Inexpensive anti-cysticercosis vaccine: S3Pvac expressed in heat inactivated M13 filamentous phage proves effective against naturally acquired *Taenia solium* porcine cysticercosis. *Vaccine*, 26, 2899–2905.
8. Assana, E., Kyngdon, C. T., Gauci, C. G., Geerts, S., Dorny, P., & De Deken, R. (2010). Elimination of *Taenia solium* transmission to pigs in a field trial of the TSOL18 vaccine in Cameroon. *International Journal for Parasitology*, 40, 515–519.
9. Rassy, D., Bobes, R. J., Rosas, G., Anaya, V. H., Brehm, K., & Hernandez, B. (2010). Characterization of S3Pvac anti-cysticercosis vaccine components: implications for the development of an anti-cestodiasis vaccine. *PLoS One*, 5, 11287–11291.
10. Morales, J., de Aluja, A. S., Martínez, J. J., Hernández, M., Rosas, G., & Villalobos, N. (2011). Recombinant S3Pvac-phage anti-cysticercosis vaccine: simultaneous protection against cysticercosis and hydatid disease in rural pigs. *Veterinary Parasitology*, 176, 53–58.
11. Olguín, S. (2010). Programa de control de la teniasis-cisticercosis. *Gaceta Biomédica*, 15, 8–9.
12. Benitez, L., Garate, T., Harrison, L. J., Kirkham, P., Brookes, S. M., & Parkhouse, R. M. (1996). Cloning and sequencing of the gene encoding the principal 18-kDa secreted antigen of activated oncospheres of *Taenia saginata*. *Molecular and Biochemical Parasitology*, 78, 265–268.
13. Harrison, G. B., Heath, D. D., Dempster, R. P., Gauci, C., Newton, S. E., Cameron, W. G., et al. (1996). Identification and cDNA cloning of two novel low molecular weight host-protective antigens from *Taenia ovis* oncospheres. *International Journal for Parasitology*, 26, 195–204.
14. Gauci, C. G., Flisser, A., & Lightowlers, M. W. (1998). *Taenia solium* oncosphere protein homologous to host-protective *Taenia ovis* and *Taenia saginata* 18 kDa antigens. *International Journal for Parasitology*, 28, 757–760.
15. Parkhouse, R. M., Bonay, P., González, L. M., Ferrer, E., Gárate, T., Aguilar, C. M., et al. (2008). TSOL18/HP6-Tsol, an immunogenic *Taenia solium* oncospherical adhesion protein and potential protective antigen. *Parasitology Research*, 102, 921–926.
16. Hernández, M., Rosas, G., Cervantes, J., Fragoso, G., Rosales-Mendoza, S., & Sciuotto, E. (2014). Transgenic plants: a 5-year update on oral antipathogen vaccine development. *Expert Rev Vaccines*, 13, 1523–1536.
17. Yusibov, V., Streatfield, S. J., & Kushnir, N. (2011). Clinical development of plant-produced recombinant pharmaceuticals: Vaccines, antibodies, and beyond. *Human Vaccine*, 7, 313–321.
18. Mihaliak, C.A., Webb, S., Miller, T., Fanton, M., Kirk, D., Cardineau, G. (2005). Development of plant cell produced

- vaccines for animal health applications. In *Proceedings of the 108th annual meeting of the United States Animal Health Association*. (pp. 158–163) Greensboro, NC.
19. Aviezer, D., Brill-Almon, E., Shaaltiel, Y., Hashmueli, S., Bartfeld, D., Mizrachi, S., et al. (2009). A plant-derived recombinant human glucocerebrosidase enzyme a preclinical and phase I investigation. *PLoS One*, *4*, 4792–4797.
 20. Hernández, M., Cabrera-Ponce, J. L., Fragoso, G., López-Casillas, F., & Guevara-García, A. (2007). A new highly effective anticysticercosis vaccine expressed in transgenic papaya. *Vaccine*, *25*, 4252–4260.
 21. Betancourt, M. A., De Aluja, A. S., Sciutto, E., Hernández, M., Bobes, R. J., Rosas, G., et al. (2012). Effective protection induced by three different versions of the porcine S3Pvac anticysticercosis vaccine against rabbit experimental *Taenia pisiformis* cysticercosis. *Vaccine*, *30*, 2760–2767.
 22. Rybicki, E. P. (2014). Plant-based vaccines against viruses. *Virology*, *11*, 1–20.
 23. Rosales-Mendoza, S., Govea-Alonso, D. O., Monreal-Escalante, E., Fragoso, G., & Sciutto, E. (2012). Developing plant-based vaccines against neglected tropical diseases: Where are we? *Vaccine*, *31*, 40–48.
 24. Luke G. (2012). Translating 2A research into practice, innovations in biotechnology, Dr. Eddy C. Agbo (Ed.) *Biochemistry, Genetics and Molecular Biology*, *8*, 168–182.
 25. Minskaia, E., Nicholson, J., & Ryan, M. D. (2013). Optimisation of the foot-and-mouth disease virus 2A co-expression system for biomedical applications. *BMC Biotechnology*, *22*, 13–67.
 26. Ryan, M. D., Donnelly, M. L. L., Lewis, A., Mehrotra, A. P., Wilkie, J., & Gani, D. (1999). A model for Nonstoichiometric, Co-translational Protein Scission in Eukaryotic Ribosomes. *Bioorganic Chemistry*, *27*, 55–79.
 27. Atkins, J. F., Wills, N. M., Loughran, G., Wu, C.-Y., Parsawar, K., Ryan, M. D., et al. (2007). A case for “StopGo”: Reprogramming translation to augment codon meaning of GGN by promoting unconventional termination (Stop) after addition of glycine and then allowing continued translation (Go). *RNA*, *13*, 803–810.
 28. Doronina, V. A., de Felipe, P., Wu, C., Sharma, P., Sachs, M. S., Ryan, M. D., & Brown, J. D. (2008). Dissection of a co-translational nascent chain separation event. *Biochemical Society Transaction*, *36*, 712–716.
 29. Ryan, M. D., & Drew, J. (1994). Foot-and-mouth disease virus 2A oligopeptide mediated cleavage of an artificial polyprotein. *The EMBO Journal*, *13*, 928–933.
 30. Ryan, M. D., King, A. M. Q., & Thomas, G. P. (1991). Cleavage of foot-and-mouth disease virus polyprotein is mediated by residues located within a 19 amino acid sequence. *Journal of General Virology*, *72*, 2727–2732.
 31. Donnelly, M. L. L., Luke, G. A., Mehrotra, A., Li, X., Hughes, L. E., Gani, D., & Ryan, M. D. (2001). Analysis of the aphthovirus 2A/2B polyprotein “cleavage” mechanism indicates not a proteolytic reaction, but a novel translational effect : a putative ribosomal “skip”. *Journal General Virology*, *82*, 1013–1025.
 32. Doronina, V. A., Wu, C., de Felipe, P., Sachs, M. S., Ryan, M. D., & Brown, J. D. (2008). Site specific release of nascent chains from ribosomes at a sense codon. *Molecular and Cellular Biology*, *28*, 4227–4239.
 33. Ryan, M. D., Luke, G. A., Hughes, L. E., Cowton, V. M., Tend-Dam, E., Xuejun, L., et al. (2002). The aphtho- and cardiovirus “Primary” 2A/2B polyprotein “Cleavage”. In B. L. Semler & E. Wimmer (Eds.), *Molecular biology of picornaviruses* (pp. 61–70). Washington, DC: ASM Press. ISBN 1-55581-210-4.
 34. Martínez-Salas, E., & Ryan, M. D. (2010). Translation and protein processing. In E. Ehrenfeld, E. Domingo, & R. P. Roos (Eds.), *The picornaviruses* (pp. 141–161). Washington, DC: ASM Press.
 35. Halpin, C., Cooke, S. E., Barakate, A., Amrani, A. E., & Ryan, M. D. (1999). Self-processing 2A-polyproteins—a system for coordinate expression of multiple protein in transgenic plants. *The Plant Journal*, *17*, 453–459.
 36. Halpin, C., Askari, B. M., Abbot, J. C., & Ryan, M. D. (2001). Enabling technologies for manipulating multiple genes on complex pathways. *Plant Molecular Biology*, *47*, 295–331.
 37. Manoutcharian, K., Rosas, G., Hernandez, M., Fragoso, G., Aluja, A., Villalobos, N., et al. (1996). Cysticercosis: identification and cloning of protective recombinant antigens. *Journal of Parasitology*, *82*, 250–254.
 38. Toledo, A., Larralde, C., Fragoso, G., Gevorkian, G., Manoutcharian, K., Hernández, M., et al. (1999). Towards a *Taenia solium* cysticercosis vaccine: an epitope shared by *Taenia crassiceps* and *Taenia solium* protects mice against experimental cysticercosis. *Infection and Immunity*, *67*, 2522–2530.
 39. Toledo, A., Fragoso, G., Rosas, G., Hernández, M., Gevorkian, G., López-Casillas, F., et al. (2001). Two epitopes shared by *Taenia crassiceps* and *Taenia solium* confer protection against murine *T. crassiceps* cysticercosis along with a prominent T1 response. *Infection and Immunity*, *69*, 1766–1773.
 40. Sambrook, J., Fritsch, E. F., & Maniatis, T. (1989). *Molecular cloning: A laboratory manual* (2nd ed.). Plainview: Cold Spring Harbor Laboratory Press.
 41. Horsch, R. B., Fry, J. E., Hoffmann, N. L., Eichholtz, D., Rogers, S. G., & Fraley, R. T. (1985). A simple and general method for transferring genes into plants. *Science*, *227*, 1229–1231.
 42. Dellaporta, S. L., Wood, J., & Hicks, J. B. (1983). A plant DNA miniprep: Version II. *Plant Molecular Biology Reporter*, *1*, 19–21.
 43. Hernández, M., Beltrán, C., García, E., Fragoso, G., Gevorkian, G., Fleury, A., et al. (2000). Cysticercosis: towards the design of a diagnostic kit based on synthetic peptides. *Immunology Letters*, *71*, 13–17.
 44. Liu, X., Constantinescu, S. N., Sun, Y., Bogan, J. S., Hirsch, D., Weinberg, R. A., & Lodish, H. F. (2000). Generation of mammalian cells stably expressing multiple genes at predetermined levels. *Analytical Biochemistry*, *280*, 20–28.
 45. Fang, J., Qian, J. J., Yi, S., Harding, T. C., Tu, G. H., Van Roey, M., & Jooss, K. (2005). Stable antibody expression at therapeutic levels using the 2A peptide. *Nature Biotechnology*, *23*, 584–590.
 46. Trichas, G., Begbie, J., & Srinivas, S. (2008). Use of the viral 2A peptide for bicistronic expression in transgenic mice. *BMC Biology*, *6*, 40–45.
 47. Urwin, P., Yi, L., Martin, H., Atkinson, H., & Gilmartin, P. M. (2000). Functional characterization of the EMCV IRES in plants. *The Plant Journal*, *24*, 583–589.
 48. Ngoi, S. M., Chien, A. C., & Lee, C. G. (2004). Exploiting internal ribosome entry sites in gene therapy vector design. *Current Gene Therapy*, *4*, 15–31.
 49. De Felipe, P., Luke, G. A., Hughes, L. E., Gani, D., Halpin, C., & Ryan, M. D. (2006). E unum pluribus: multiple proteins from a self-processing polyprotein. *Trends in Biotechnology*, *24*, 68–75.
 50. Ha, S. H., Liang, Y. S., Jung, H., Ahn, M. J., Suh, S. C., Kweon, S. J., et al. (2010). Application of two bicistronic systems involving 2A and IRES sequences to the biosynthesis of carotenoids in rice endosperm. *Plant Biotechnology Journal*, *8*, 928–938.
 51. Rasala, B. A., Lee, P. A., Shen, Z., Briggs, S. P., Mendez, M., & Mayfield, S. P. (2012). Robust expression and secretion of Xylanase1 in *Chlamydomonas reinhardtii* by fusion to a selection gene and processing with the FMDV 2A peptide. *PLoS One*, *7*, 433–449.