

RESEARCH ARTICLE

# The Corn Smut ('Huitlacoche') as a New Platform for Oral Vaccines

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## Abstract

The development of new alternative platforms for subunit vaccine production is a priority in the biomedical field. In this study, *Ustilago maydis*, the causal agent of common corn smut or 'huitlacoche' has been genetically engineered to assess expression and immunogenicity of the B subunit of the cholera toxin (CTB), a relevant immunomodulatory agent in vaccinology. An oligomeric CTB recombinant protein was expressed in corn smut galls at levels of up to 1.3 mg g<sup>-1</sup> dry weight (0.8% of the total soluble protein). Mice orally immunized with 'huitlacoche'-derived CTB showed significant humoral responses that were well-correlated with protection against challenge with the cholera toxin (CT). These findings demonstrate the feasibility of using edible corn smut as a safe, effective, and low-cost platform for production and delivery of a subunit oral vaccine. The implications of this platform in the area of molecular pharming are discussed.

## Introduction

Following infection of young maize seedlings with the fungus *Ustilago maydis*, tumors or galls are formed in all aerial parts of these plants, and these galls are known as corn smut or 'huitlacoche' [1–2]. Interestingly, 'huitlacoche', also known as Mexican corn truffles, has been consumed by populations in Mexico since pre-Colombian times. Since then, its edible popularity has gained wider acceptance, and has expanded to other countries where it is now consumed as an exotic delicacy.

A number of studies have revealed that 'huitlacoche' has a high nutritional value, as it is high in protein and in essential amino acids, such as lysine, an amino acid that is commonly deficient in corn protein. Compared to other edible fungi, 'huitlacoche' has a higher content of bioactive compounds with antitumoral activities, such as  $\beta$ -glucans, thus serves as a viable functional food candidate [1, 3]. Additionally, *U. maydis* produce secondary metabolites

having valuable applications, such as glycolipids (cellobiose lipid-ustilagic acid and mannosylerythritol lipids-MEL), siderophores, indole pigments, and protease [4].

*U. maydis* is a hemibasidiomycete with a narrow host range, as it only infects maize (*Zea mays*) and its progenitor plant teosinte (*Zea mays* subsp. *parviglumis*). The sexual stage of *U. maydis* begins when compatible haploid sporidia mate on young leaf tissues, establishing dikaryotic filaments that invade these tissues, and inducing tumor formation [5]. Within these tumorous tissues, diploid teliospores with thickened cell walls are produced, which in turn can germinate to initiate the next generation cycle [6]. Development of the dikaryon is controlled by the biallelic *a* mating type locus, encoding for the pheromone precursor (*mfa1/2*) and the receptor (*pra1/2*), that regulates cell fusion [7]. Subsequently, the bE/bW transcription factor, encoded by the multiallelic *b* locus and formed via the interaction of the homeodomain proteins bE and bW, controls the pathogenic stage of this fungus [8]. Thus, only haploid cells with different *a* and *b* loci can mate and produce infectious filaments.

Availability of genomic resources, including a genome sequence, plasmids, selectable markers, constitutive and regulated promoters, critical reporters, along with a relatively short duration to obtain infected plants [9–11] have all contributed to increased knowledge of the biology of *U. maydis*, rendering it as a useful and ideal model organism for pursuing cellular and molecular studies. Recently, it has been suggested that *U. maydis* in its free life form should be considered for expression of recombinant proteins. In this stage, *U. maydis* grows as haploid yeast-like cells that are easily manipulated, thus allowing them to grow in a variety of low-cost production systems, even as substrates in biological waste products [12].

Recombinant organisms providing edible biomass would serve as attractive systems for both expression and delivery of high-value proteins such as subunit vaccines [13–14]. Production of low-cost vaccines that can be administered orally can have a significant impact on world-wide mass immunization efforts [15–16]. Although filamentous fungi are robust systems for the production of recombinant proteins [17], their use for vaccine production has not yet been fully explored. The B subunit of the cholera toxin (CTB) is a potent immunogen associated with protection against *Vibrio cholera* and enterotoxigenic *Escherichia coli* (ETEC) [18]. In addition, it serves as a mucosal adjuvant, as it is used as a carrier for mucosal delivery of chemically- and genetically- coupled antigens [19]. Immunization through oral or nasal administration of CTB-coupled antigens has demonstrated therapeutic immune modulatory activity, even in models of autoimmune diseases [20]. The immunogenicity of CTB is associated with the correct pentamer formation that binds to the GM1 receptor, present in most nucleated cells [19, 21].

In this study, expression of the B subunit of the cholera toxin (CTB) was achieved in corn smut ('huitlacoche'), following infection of maize with transgenic *U. maydis* strains carrying the *CTB* gene, in an effort to explore the feasibility of using corn smut or 'huitlacoche' as a novel host for production and delivery of safe oral subunit vaccines. This will contribute to an innovative production platform for vaccines and for other therapeutic compounds.

## Materials and Methods

### Strains and growth conditions

The wild-type strains of *U. maydis*, FB1 (*a1b1*) and FB2 (*a2b2*), and the FB2-derivative strains were grown routinely in either liquid YPD (2% yeast extract, 1% peptone, and 1% glucose) or YEPSL (0.4% yeast extract, 0.4% peptone, and 2% sucrose) medium at 28°C, along with continuous agitation on a rotary shaker at 200 rpm. For cloning of the pMF1h-CTB plasmid, *E. coli* Top10 strain was used.

## DNA and RNA isolation

Total DNA and RNA from *U. maydis* strains and from corn smut galls (teliospores) were isolated as previously described [22–23], respectively.

## Plasmid construction, transformation, and analysis

In order to generate a *Po2tef::CTB* fusion, the open reading frame (ORF) of the cholera toxin B subunit (CTB) was amplified using the vector pUC57-CTB as template, carrying a synthetic CTB gene optimized for expression in eukaryotic cells. The following primers were used: CTB<sub>fwd</sub> 5′-TGAGGATCCATGATCAAGCTTAAGTTCGGAGTTTTTC-3′ (sense) and CTB<sub>rev</sub> 5′-TAGGCGCGCCTCATCCAGAGGAGTTACCGGAA-3′ (antisense), wherein the *Bam*HI and the *Asc*I restriction sites were included, respectively. The constitutive promoter *Po2tef* was amplified from the pMF2-3c plasmid [9] using the primers pOTEF<sub>fwd</sub> 5′-TGGGTACCAAC TAGTGGATCCCCGTAC-3′ (sense) and pOTEF<sub>rev</sub> 5′-AGTCAGAATTCCTCACTCAGGCC TATTATGCC-3′ (antisense) with the *Kpn*I and *Eco*RI restriction sites, respectively. Subsequently, amplicons were digested, and then ligated. Using primers pOTEF<sub>fwd</sub> and CTB<sub>rev</sub>, the ligation product was diluted 1:10, and used as a template to obtain the *Po2tef::CTB* PCR product. The PCR product was digested with *Kpn*I and *Asc*I restriction enzymes, cloned into corresponding restriction sites of the pMF5-1h plasmid, thus replacing the *egfp* coding sequence, and fusing the digested amplicon upstream of the *nos* terminator.

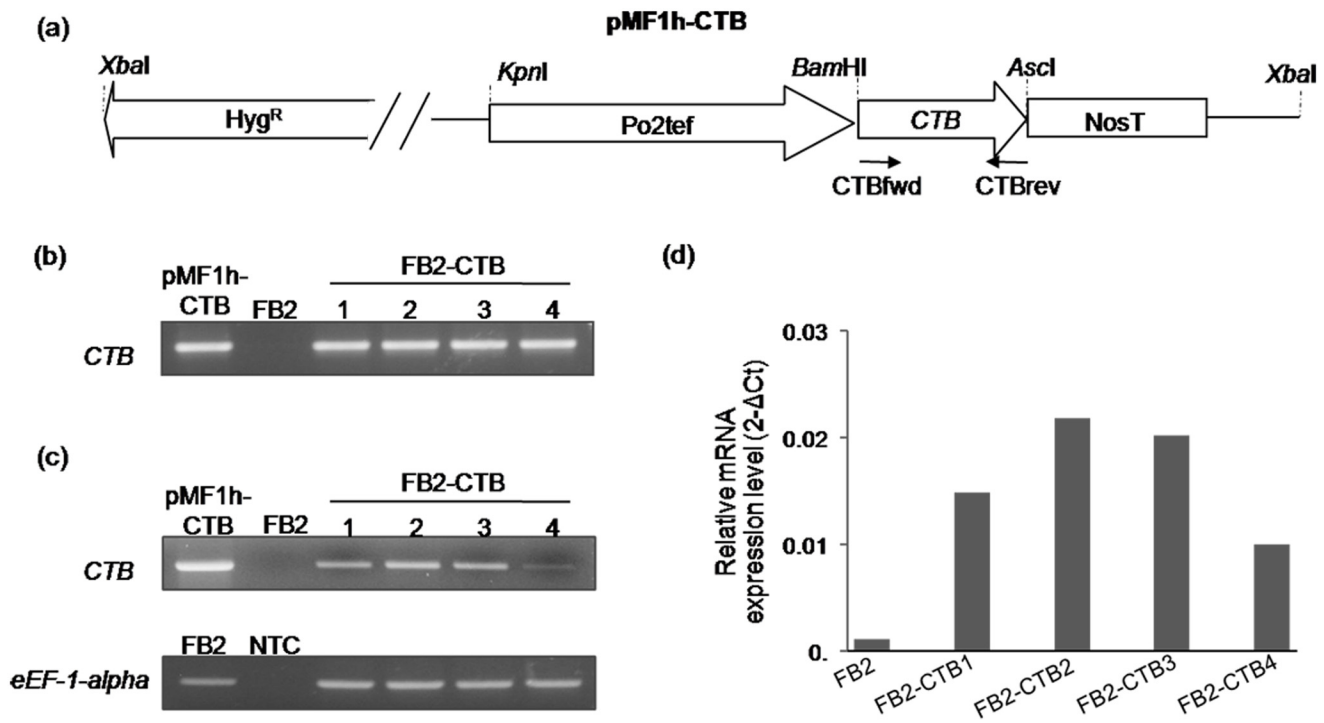
Following sequencing, the resulting plasmid, designated as pMF1h-CTB (Fig 1a), was linearized with an *Xba*I restriction enzyme, and then used to transform *U. maydis* FB2 protoplasts as previously described [9]. Cells were grown in YEPSL medium at an OD<sub>600</sub> of 1.0. After harvest, cells were washed once with SCS [20 mM sodium citrate (pH 5.8), 1 M sorbitol], and resuspended in 2 ml of SCS containing 10 mg/ml of the lytic enzyme of *Trichoderma harzianum* (Sigma-Aldrich). The mix was incubated for 30 min at room temperature for fungal cell wall digestion. The reaction was stopped by adding an SCS buffer, and then washed twice with SCS. Finally, cells were washed once with STC [10 mM TRIS-HCl (pH 7.5), 100 mM CaCl<sub>2</sub>, and 1 M sorbitol], and cells were resuspended in STC.

After adding 5 μL of linearized DNA (1 μg/μL) and 1.5 μL of heparin 1 (1.0 mg/mL), 50 μL of protoplasts were incubated for 10 min in ice. Then, 500 μL of STC/PEG (10 g of PEG4000 in 15 ml of STC) was added, and the mixture was incubated for 15 min on ice. Briefly, cells were spread in 10 mL of regeneration agar (YEPSL with 1.5% agar, 1 M sorbitol and 400 μg/mL hygromycin B), and overlaid with 10 mL of regeneration agar without antibiotic.

Integration of the plasmid was confirmed by PCR amplification of the CTB gene using the CTB<sub>fwd</sub> and CTB<sub>rev</sub> primers, and genomic DNA from each of the *U. maydis* strains was used as template. The following four transgenic lines were identified, and designated as FB2-CTB 1, 2, 3, and 4 (*a2b2 Po2tef::CTB*).

## CTB gene expression

Total RNA was isolated from cells of four transgenic strains of *U. maydis* grown for 12 h in YPD medium and from corn smut galls harvested 18 days post-infection (dpi). Following treatment with DNase I (Promega), first-strand cDNA synthesis was performed in a total volume of 20 μL using 2 μg of total RNA with the SuperScript II Reverse Transcriptase (Invitrogen, CA, USA) following the manufacturer's instructions. PCR was carried out in a 25 μL reaction with 1 μL of RT reaction product used as a template and CTB<sub>fwd</sub> and CTB<sub>rev</sub> specific primers. PCR conditions were as follows: 94°C for 5 min, 30 cycles of 94°C for 30 s, 57°C for 30 s, 72°C for 40 s, followed by a final extension of 72°C for 5 min. PCR products were electrophoretically separated on 2% agarose gels, and visualized following ethidium bromide staining. A 140 bp



**Fig 1. Expression of the *Vibrio cholerae* CTB gene in the yeast-like *U. maydis* FB2 strain.** (a) Schematic diagram of the pMF1h-CTB plasmid used for transformation of *U. maydis* FB2. The *CTB* gene was fused to the constitutive *Po2tef* promoter and to the nopaline synthase terminator (*NosT*). (b) Specific amplification of the *CTB* gene from the genomic DNA of four FB2-CTB transformants, using the *CTBfwd* and *CTBrev* primers. (c) and (d) Expression of the *CTB* gene in four FB2-CTB transformants determined by semi-quantitative RT-PCR and quantitative RT-PCR expression, respectively. Quantitation of the *CTB* gene expressed as relative mRNA expression levels ( $2^{-\Delta Ct}$ ), was calculated after normalization to the *U. maydis eEF-1-alpha* gene, used as control. Total RNA was isolated from WT and *CTB*-expressing FB2 yeast-like cells grown in YPD medium in agitation at 28°C, during 12 h. Genomic DNA from *U. maydis* FB2 strain, the plasmid pMF1h-CTB, and a sample without template (NTC), were used as control.

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RT-PCR product of the elongation factor gene *eEF-1-alpha* (um00924) was used as a control [24]. For quantitative RT-PCR analysis, total RNA extracted from *U. maydis* strains or corn smut galls was treated with DNase I (Invitrogen), and the amount was determined before and after DNase treatment using a NanoDrop ND-1000 UV-VIS spectrophotometer (NanoDrop Technologies). cDNA synthesis and quantitative PCR analyses were done in a 10  $\mu$ L reaction mixture containing 50 ng of total RNA as template, using the Power SYBRGreen RNA-to-CT1-Step Kit (Applied Biosystems) [25]. The primers used were the qCTBfwd 5' -CGCTTATCTTACAGAAGCTAAGG-3' (sense) and the CTBrev (antisense). Quantification was based on a cycle threshold value wherein the expression level of the *CTB* gene was normalized to transcript levels of the *U. maydis* elongation factor *eEF-1-alpha* (um00924) [24].

### Mating and maize infection assay

To evaluate the effects of *CTB* gene expression on mating capacities of FB2-CTB transformants, independent compatible strains were grown overnight, and adjusted to an OD<sub>600</sub> of 1.0 in sterilized water. Crosses of paired strains were made, 1:1 ratio (v/v), and 4  $\mu$ L of each cross or strain were inoculated in a minimal medium containing 1% charcoal [26]. Culture plates were sealed, and incubated at room temperature for 48 h.

To assess pathogenicity of transgenic *U. maydis*, 8 day-old greenhouse-grown seedlings of creole *Zea mays* (cv. Morado Pardo) were infected by injecting fungal crosses into leaf whorls

with the following crosses: FB1 x FB2, FB1 x FB2-CTB 3, and FB1 x FB2-CTB 4. Disease symptoms were scored from 8 to 12 dpi.

Maize ear galls were induced by injecting 10 mL of each strain mixture into silk channel of primary ears, 4–6 days after silking. All plants were maintained under greenhouse conditions, and disease symptoms were scored 15 to 20 dpi. Samples of ‘huitlacoche’ were harvested 18–20 dpi, lyophilized, and then pulverized in a miller analytical.

## CTB immunodetection and quantification

Expression of CTB was assessed using Western blot assays. Protein extracts were obtained by resuspending 30 mg of freeze-dried tissue of corn smut galls in 300  $\mu$ L of the extraction buffer [750 mM Tris-HCl (pH8.0), 15% (w/v) sucrose; 100 mM  $\beta$ -Mercaptoethanol; and 1 mM PMSF [27] following centrifugation at 12,000 rpm for 15 min and at 4°C. The supernatant was kept, and 30  $\mu$ L of protein extracts were mixed with the same volume of 1X reducing loading buffer. Samples were denatured at 95°C for 5 min, and SDS-PAGE was performed in 4–12% polyacrylamide gels. The gel was blotted onto a BioTrace PVDF membrane (Pall Corporation), and subjected to blocking in phosphate saline buffer (PBS) along with 0.01% Tween 20 (PBST) plus 5% fat-free milk. Primary labelling was performed overnight using a mouse anti-CTB antiserum (1:200 dilution). A horseradish peroxidase-conjugated secondary anti-mouse antibody (1:2,000 dilution, Sigma, St. Louis, MO, USA) was added, and the membrane was incubated for 2 h at room temperature. Antibody detection was performed by adding the SuperSignal West Dura solution following the manufacturer’s instructions (Thermo, MA, USA), and signals were detected on an X-ray film following standard procedures. Distinct amounts of pure CTB were included as positive controls (100, 250 and 500 ng per well; Sigma, St. Louis, MO, USA).

## GM1 binding assay

To quantify the amount of protein present in freeze-dried ‘huitlacoche’ biomass, protein extractions were performed using 30 mg of dry biomass resuspended in 300  $\mu$ L of protein-extraction buffer [50 mM Tris-base (pH 8.0), 40 mM NaCl, 0.5% Triton X-100 (v/v), and 1 mM PMSF]. After vortexing, samples were centrifuged at 14,000 rpm for 15 min at 4°C. Total soluble protein was quantified using the Lowry method using BSA as a standard [28]. CTB content in the soluble protein fraction was determined using the GM1 ganglioside dependent ELISA assay [29]. Briefly, plates were coated with Type III GM1 ganglioside (1.5  $\mu$ g per well, SIGMA) for a period of 1 h. After washing with PBST, plates were blocked for 1 h at room temperature with 5% fat-free milk, dissolved in PBST. Protein extracts were added, and plates were incubated overnight at 4°C. Primary labelling was performed overnight with a mouse anti-CTB antiserum (1:200 dilution). Horseradish peroxidase-conjugated secondary anti-mouse antibody (1:2000 dilution, SIGMA) was applied for 2 h at room temperature. Color reaction was detected by adding an ABTS substrate (SIGMA). Optical density values were measured at OD405nm using a Multiskan Ascent microplate reader (Thermo Electron Corporation, Waltham, MA, USA) and used to quantify CTB yields as percentages of the total soluble protein (% TSP), using a standard curve prepared with known amounts of the pure CTB protein (0.02–0.2 ng/ $\mu$ L; Sigma).

## Immunogenicity assay

This immunogenicity assay was carried out in accordance with the recommendations listed in the Guide for Care and Use of Laboratory Animals of the National Institute of Health. The protocol was approved by the Committee on Research Ethics of the Chemistry College/University of San Luis Potosí (Permit Number: CEID-2013-004). All efforts were made to minimize animal suffering.



Two groups ( $n = 4$ ) of 12 week-old female BALB/c mice were randomly established, and subjected to oral administration with one of the following two treatments, 50 mg of freeze-dried FB2-CTB 3 galls (containing 65  $\mu\text{g}$  of oligomeric CTB) or 50 mg of freeze-dried WT galls. Doses were prepared by resuspending the gall powder in 100  $\mu\text{l}$  PBS, and administered on days 0, 7, and 14. Mice were bled at day 21, and sera were collected to determine antibody levels using ELISA analysis [30]. Ninety six-well polystyrene plates were coated overnight with CTB at 0.25  $\mu\text{g}/\text{well}$  at 4°C. After blocking with 5% fat-free milk for 2 h, plates were further incubated overnight at 4°C with serial dilutions of mice sera in PBS (1:10 to 1:80 dilutions). Horseradish peroxidase-conjugated secondary anti-mouse antibody (1:2000 dilution, Sigma, St Louis, MO, USA) was applied for 2 h at room temperature, and after washing, signals were detected following incubation with an ABTS substrate for 15 min (SIGMA). Specific antibody levels were expressed as corresponding optical density values measured at OD<sub>405nm</sub> using a Microplate reader (Thermo Electron Corporation). IgG titers were then calculated as follows: the reciprocal of the higher serum dilution with an OD value above the mean OD value of the WT group plus 2 times its standard deviation.

The Cholera toxin (CT) challenge was performed on day 28 following the procedure previously described [31], but with some modifications [30]. In addition to the two experimental groups treated with either WT or transgenic 'huitlacoche', two control mice groups were included. One control consisted of an unchallenged mice group, while a second control consisted of a naïve mice group treated with PBS and challenged with CT. Briefly, either 10  $\mu\text{g}$  of CT diluted in 10% NaHCO<sub>3</sub> or the vehicle alone were orally administered to food-deprived mice, wherein food has been withheld for a period of 16 h. After a period of 6 h, with access only to water, mice were sacrificed by cervical dislocation, and the entire small intestine, from the pyloric valve to the ileal-cecal junction, was dissected, and weighed. The volume of fluid accumulation (FA) in the small intestine, following challenge, was calculated as described [31], and according to the following:  $\text{FA} = (G/B - G) \times 1000$ ; wherein  $B$  is total body weight (in grams) and  $G$  is total gut weight (including fluids) (in grams).

## Statistical analysis

ELISA data presented correspond to the geometric means of values obtained per group, while error bars represent standard deviations. Significant differences in antibody levels and fluid accumulation values between pairs of groups are assessed using one-way analysis of variance followed by mean comparisons using Tukey's test with  $P$  values  $< 0.05$  deemed significant.

## Results

### Developing *U. maydis* strains over-expressing the CTB gene

In order to express the B subunit of the cholera toxin of *V. cholerae*, several transgenic *U. maydis* strains overexpressing the CTB gene, which has been optimized for expression in eukaryotic cells, under the control of the constitutive and strong *Po2tef* promoter were generated (Fig 1a). This promoter is a chimeric fusion of seven tetracycline responsive elements to the basal promoter of the translation elongation factor 2 from *U. maydis* [32]. The pMF1h-CTB plasmid was introduced into *U. maydis* FB2 strain, and four hygromycin-resistant transformants, designated as FB2-CTB 1, 2, 3, and 4, were found to carry the CTB transgene following PCR amplification of the full-length ORF, while no amplification was detected in the FB2 WT (Fig 1b). Moreover, expression of CTB in transformed strains was confirmed by semi-quantitative and quantitative RT-PCR (Fig 1c and 1d).

## Production of 'huitlacoche' expressing the CTB gene

Prior to inoculation of maize with transgenic *U. maydis* FB2-CTB strains, mating capacities of these strains were evaluated. This is a prerequisite for dikaryon establishment, and for pathogenic development. Each of the four transgenic strains, FB2-CTB 1, 2, 3, and 4, were co-spotted with the mating FB1 partner on charcoal-MM plates, and then incubated at room temperature for 48 h. All transgenic FB2-CTB strains developed fuzzy phenotypes, along with white colonies, that were indistinguishable from those of compatible WT strains (Fig 2). This finding suggested that FB2-CTB 1, 2, 3, and 4 strains were competent to mate with the FB1 WT strain.

To assess the pathogenicities of the strains, 8-day-old maize seedlings were inoculated with mixtures of *U. maydis* FB1 x FB2-CTB 3 and FB1 x FB2-CTB 4 transgenic strains, and with the control FB1 x FB2 strains. It was found that FB1 x FB2-CTB 3 and FB2-CTB 4 mixtures induced tumor development after 8 dpi, which was similar to that observed for the FB1 x FB2 mixture (Fig 3a–3c). Furthermore, transgenic FB2-CTB 3 and FB2-CTB 4 paired with FB1 induced tumors on inoculated ear cobs, and these tumors were indistinguishable from those generated following inoculation with the FB1 x FB2 cross (Fig 3d–3f and S1 Table). However, in contrast to WT 'huitlacoche', transgenic corn smut galls expressed the CTB gene (Fig 4a).

## Immunodetection and quantification of the oligomeric recombinant CTB protein of 'huitlacoche'

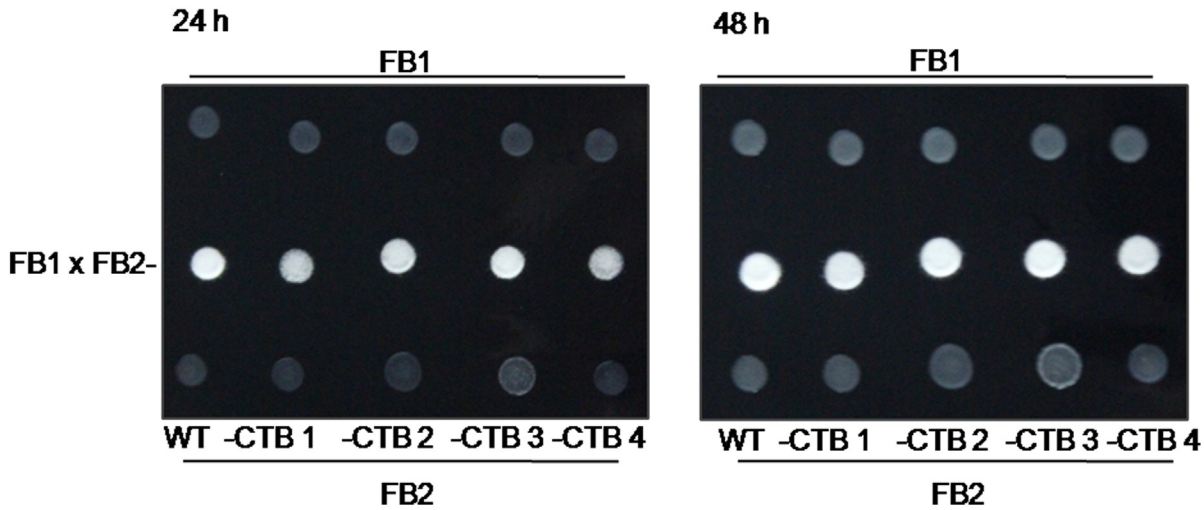
To assess the presence and integrity of the CTB protein in transgenic 'huitlacoche', Western Blot analysis was conducted using anti-CTB serum. Extracts of 'huitlacoche' infected with mixtures of FB1 x FB2-CTB 3 and 4 showed strong signals of 12 kDa in molecular weight, 3 kDa lower than that of the predicted molecular weight of the CTB protein expressed in *U. maydis* (15 kDa). As expected, wild-type corn galls did not show any signals. In spite of the reducing conditions and boiling temperatures of the assay, a 24 kDa band was also detected, likely corresponding to dimers of the CTB protein (Fig 4b). Moreover, a quantitative GM1-ELISA assay revealed that levels of the oligomeric CTB reached up to 1.3 mg g<sup>-1</sup> dry weight of gall tissues, corresponding to 0.8% TSP. This finding also demonstrated the proper assembly of the oligomeric structure of CTB, which is critical for its immunogenic activity as binding to GM1 is dependent on this structure and its biological activity (Fig 4c).

## The corn smut CTB recombinant protein confers protection against CT challenge

Following a three-week immunization scheme, mice orally immunized with transgenic FB1 x FB2-CTB 3 'huitlacoche' showed significantly higher intestinal IgA and serum IgG levels than those immunized with WT 'huitlacoche' (Fig 5a and 5b). The anti-CTB IgG titer for the FB1 x FB2-CTB 3 'huitlacoche'-immunized group was 40. When mice were challenged with the cholera toxin (CT), fluid accumulation (FA) levels were significantly lower (mean of 51.8) for mice immunized with transgenic 'huitlacoche' than those immunized with wild-type tissues (mean of 93.1). In contrast, FA levels of unchallenged mice showed a mean of 52.2 (Fig 6). The observed protective effects induced by the CTB protein produced in transgenic 'huitlacoche'-suggested elicitation of a humoral response in intestinal mucosal tissues in mice.

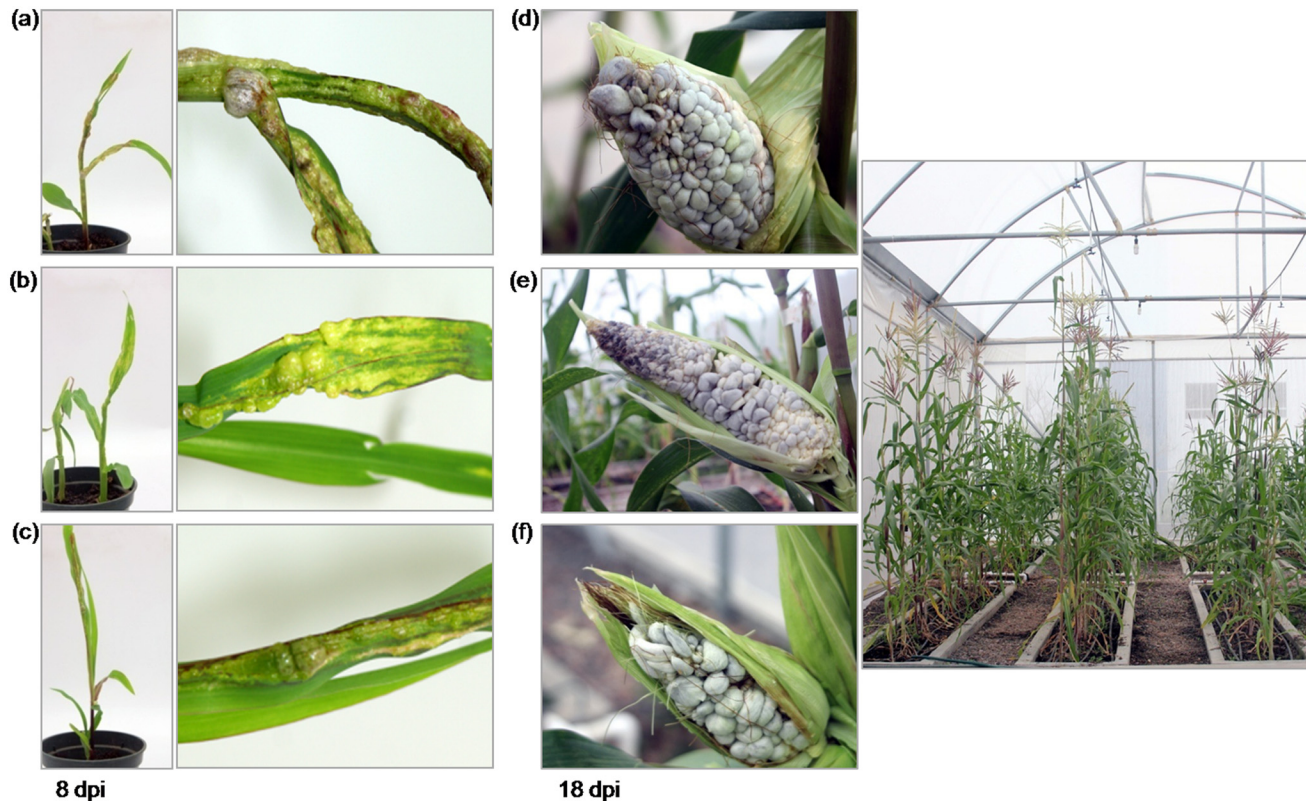
## Discussion

The CTB protein, as well as various other antigenic proteins, has been expressed in a number of organisms/hosts, including bacteria, yeast, plants (tobacco, tomato, lettuce, and maize), and green algae, *Chlamydomonas reinhardtii* [16, 33–35]. In some systems, either a lack of proper



**Fig 2. *U. maydis* FB2-CTB strains develop fuzzy colonies when are paired with the FB1 strain.** FB2-CTB transformants were co-spotted with their mating partner FB1 strain on a charcoal-containing MM plates. The FB2 x FB1 cross was tested as control. Photos were taken 24 and 48 h after incubation at room temperature.

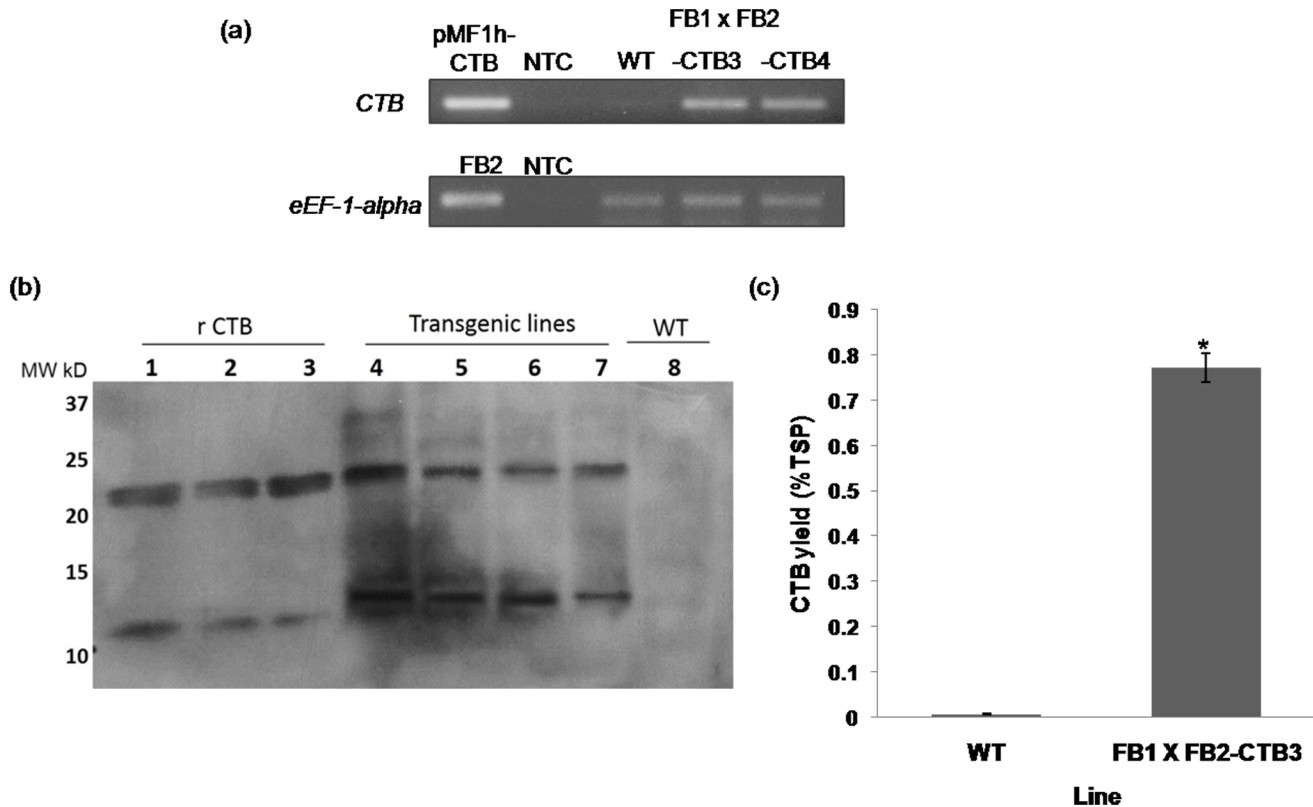
doi:10.1371/journal.pone.0133535.g002



**Fig 3. Tumors induced on maize plants, after inoculation with FB2-CTB strains paired with FB1 strain.** Leaf tumors induced in 8-day-old maize seedlings inoculated with (a) FB1 x FB2, (b) FB1 x FB2-CTB 3 and (c) FB1 x FB2-CTB 4 crosses. Photos were taken 8 days post-inoculation (dpi). Corn smut galls ('huitlacoche') produced by inoculating with (d) FB1 x FB2, (e) FB1 x FB2-CTB 3 and (f) FB1 x FB2-CTB 4 crosses. Plants were grown under greenhouse conditions and photos were taken 18–20 dpi.

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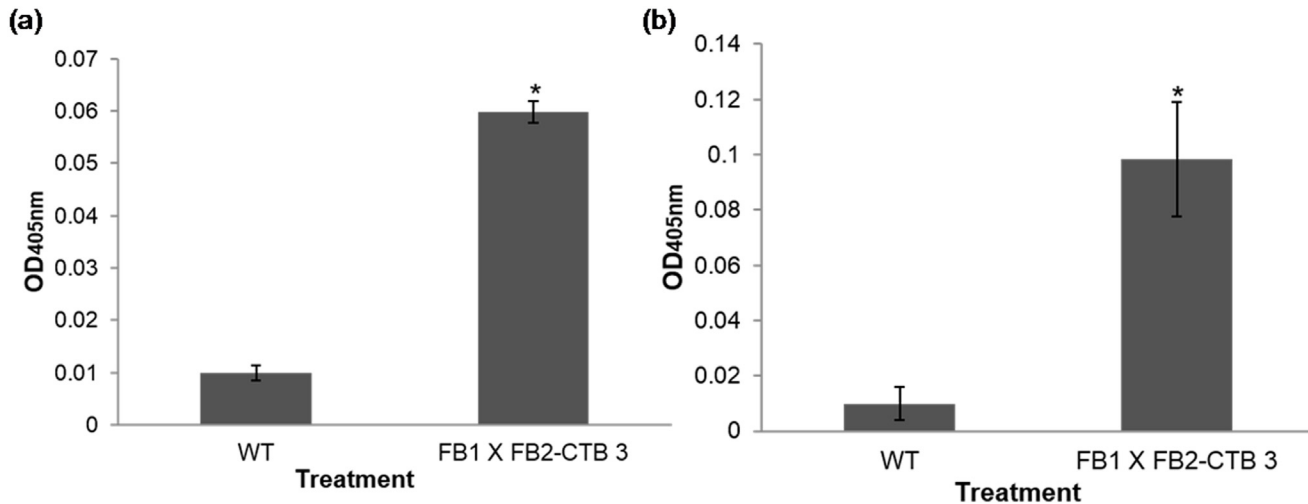


**Fig 4. *V. Cholerae* CTB gene is successfully expressed in corn smut galls ('huitlacoche').** Corn cobs were inoculated with the crosses FB1 x FB2-CTB 3 and FB1 x FB2-CTB 4, and FB1 x FB2 as control. (a) Expression analysis of the CTB gene in 'huitlacoche' by semi-quantitative RT-PCR. Total RNA was isolated from fresh WT or transgenic corn smut galls (teliospores). Genomic DNA from *U. maydis* FB2 strain, the plasmid pMF1h-CTB, and a sample without template (NTC), were used as control. (b) Detection of the CTB protein in corn smut galls protein extracts by Western Blot analysis. Protein extracts were subjected to SDS PAGE under reducing conditions, blotted, and then labelled with an anti-CTB serum. Lanes 1 to 3, pure CTB standard (500, 250, and 100 ng, respectively); 4 and 5, extracts from 'huitlacoche' produced with the FB1 x FB2-CTB3 cross; 6 and 7, 'huitlacoche' produced with the FB1 x FB2-CTB 4 cross; and 8, extract from WT 'huitlacoche' (FB1 x FB2). (c) Quantification of CTB levels in terms of %TSP by GM1-ELISA assay. Oligomeric CTB was detected in total soluble protein extracts from either FB1 x FB2-CTB 3 galls or WT galls followed by immunodetection with an anti-CTB serum. The Asterisk denotes statistical differences at  $P < 0.05$  versus WT galls.

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assembly or undesirable addition of a glycan moiety has been reported [35], [36]. Therefore, identifying an alternative platform for production and expression of CTB along with other antigenic proteins that encounter similar problems of expression in host systems is highly desirable.

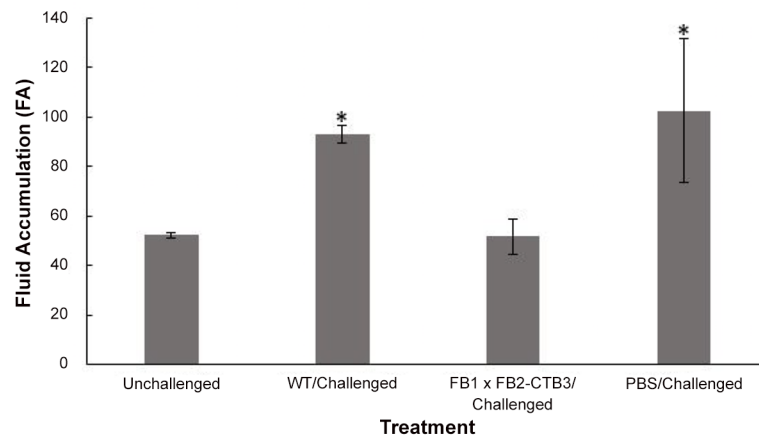
To develop a low-cost subunit vaccine production platform, we have exploited corn smut galls or 'huitlacoche' for production and delivery of a therapeutic recombinant protein as a novel and unique strategy. The CTB protein was produced in corn smut galls by infecting corn ears with a mixture of compatible haploid cells of *U. maydis* wherein the mating partner FB2 alone is carrying the CTB gene. This has been designed as a biosafety measure, although *U. maydis* only infects maize and not humans. Moreover, 'huitlacoche' expressing CTB was produced under greenhouse conditions to prevent gene flow and control the release of the transgene, and to optimize the environmental plant growth conditions, including irrigation, as drought is one of the most important limiting conditions for maize production [37]. Developing healthy plants contribute to high amounts of biomass production and protein accumulation, both critical for large-scale molecular farming of therapeutic proteins [38].



**Fig 5. Antibody levels of BALB/c mice immunized with corn smut galls expressing CTB protein.** Detection of: (a) anti-CTB IgA antibodies in intestinal washes, and (b) anti-CTB IgG antibodies in sera. Mice immunized with different treatments (biomass from ‘huitlacoche’: FB1 x FB2-CTB 3 and FB1 x FB2 as control) were bled at day 21, and serum was diluted 1:40. Statistical differences versus the WT group are indicated by an asterisk ( $P < 0.05$ ).

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Herein, cloning and expression of the *CTB* gene in yeast-like *U. maydis* FB2 strain, and subsequent production of the proper structure of the CTB recombinant protein in ‘huitlacoche’ has been successfully achieved. The ‘huitlacoche’ produced after inoculation with different transgenic strains has been identified to express varying levels of the oligomeric CTB protein, reaching yields of up to 1.3 mg g<sup>-1</sup> dry weight of galls (0.8% TSP). This demonstrates the high biosynthetic capacity of *U. maydis* as many other fungal species [39]. In the molecular farming arena, recombinant protein expression in seeds and viral expression systems are the most



**Fig 6. Immunoprotection against CT challenge of BALB/c mice immunized with galls expressing the CTB protein.** Mice were immunized with either WT galls or FB1 x FB2-CTB 3 galls, and then challenged with CT. Control groups consisted of an unimmunized mice group challenged with CT (PBS/challenged) and an unimmunized unchallenged mice group (unchallenged). Mice were challenged with 10 μg of CT one week following the last boost. After 6 h of CT toxin administration, mice were sacrificed, and fluid accumulation (FA) was estimated by weighing carcass and small intestines. Comparisons between challenged and unchallenged groups have been made, and significant differences found against the unchallenged group are denoted using an asterisk ( $P < 0.05$ ). Note the lower FA values in the group immunized with FB1 x FB2-CTB 3 galls.

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promising platforms in terms of yields. Although viral expression vectors have allowed for recovery of very high yields of CTB (up to  $>1 \text{ g kg}^{-1}$  fresh weight) in leaves of *Nicotiana benthamiana*, this expression platform requires purification of the recombinant protein as it is not an appropriate vehicle for oral delivery, due to the presence of toxic compounds [35]. Several efforts to produce CTB or chimeric CTB-based proteins in seed crops have been performed, mainly in rice and maize. In rice, highest yields of chimeric CTB of  $1.5 \mu\text{g g}^{-1}$  dry seed have been reported [40], while  $50 \mu\text{g g}^{-1}$  dry seed yields were reported by [41]. In maize, expression of CTB has reached up to  $1.56 \mu\text{g g}^{-1}$  dry weight [42]. The CTB yields obtained in the 'huitlacoche' system are very high when compared to those reported in seed crops.

This is the first report on the use of 'huitlacoche' (corn smut) for the production of subunit vaccines. Although long-term storage at room temperature is a major advantage of expression of recombinant therapeutic proteins in maize seeds, serious concerns over gene flow due to wide-range spread of pollen and lengthy process of developing stable transgenic maize lines render this system less desirable [38, 43]. The 'huitlacoche' system offers a suitable model to overcome these drawbacks and concerns as it is produced after infecting corn with mixtures of compatible *U. maydis* haploid sporidia that are easily transformed. Additionally, the production of purified recombinant proteins from yeast-like *U. maydis* grown in batch cultures is also feasible.

Interestingly, the molecular weight of the recombinant protein (12 kDa) is found to be lower than predicted (15 kDa), suggesting that the peptide leader sequence of CTB, first 21 amino acids, has been removed. Previously, it has been suggested that the peptide leader sequence of the CTB protein is involved in translocation of the protein to the endoplasmic reticulum (ER) of eukaryotic systems, wherein proper assembly of the recombinant protein takes place in spite of its glycosylation [35, 36, 44]. Additionally, the GM1 binding assay of CTB produced in 'huitlacoche' indicates that the pentameric structure of CTB has been properly assembled in this tissue. As glycosylation is a relevant post-translational modification, this influences immunogenicity, function, and half-life of recombinant protein, and serves as a major challenge in heterologous expression systems. First, undesired glycosylation of antibodies and hormones may become immunogenic leading to undesired immune responses resulting in tissues damage [45]. Second, glycosylation patterns in target antigens might enhance immunogenicity of the candidate vaccine, and accounting for vaccine efficacy [46]. Previously, it has been reported that *U. maydis* has an unconventional secretory system that prohibits transfer of proteins through ER [47]. Thus, this serves as a convenient system for expression of proteins that do not require this modification. However, expression of glycosylated proteins is also suitable as the glycosylation pathway of *U. maydis* seems to be more similar to that of mammals than that of yeasts as *Saccharomyces cerevisiae* [48].

More importantly, oral immunization of BALB/c mice with transgenic 'huitlacoche' has revealed that the 'huitlacoche'-produced recombinant CTB protein elicits a high level of immunogenicity in test mice. Although immunogenicity of recombinant CTB protein in other host systems has been reported, the high level of immunogenicity observed in 'huitlacoche' is highly desirable. Furthermore, the protective potential of the elicited immune response, determined in CT-challenged immunized mice and measured by FA values, has demonstrated the neutralizing activity of the recombinant CTB protein in 'huitlacoche'. In addition, assessment of the immune protection in *V. cholera* or ETEC infection models will provide valuable and critical information for use as a candidate vaccine for prevention of these diarrheal diseases that are highly prevalent in developing countries [49]. As CTB is also recognized as a tolerogenic immunogen associated with the expansion of regulatory T cells, the use of this expression system for developing subunit vaccines against autoimmune diseases will contribute to new research thrusts [50]. Future studies should pursue determination of the effects of distinct formulations of this recombinant CTB

on immunogenicity. For example, the effects of particle size or the use of gelatin pills to ensure proper dosage are pertinent research objectives. In addition, studying the effect of thermic treatments, e.g. pasteurization, on the vaccine shelf life would be relevant.

To our knowledge, filamentous fungi have not been extensively explored as a platform for the production of recombinant proteins, thus, this study provides a new model of edible fungal species that can be used as a biofactory for production of therapeutic proteins, such as subunit vaccines. The 'huitlacoche' biomass also serves as an economic and safe production system for an oral subunit vaccine and at low-cost, as purification of the recombinant protein is not necessary.

## Supporting Information

**S1 Table. Yield of 'huitlacoche' produced after inoculation of corn cobs with the *U. maydis* F1 x FB2-CTB 3 and the FB1 x FB2-CTB 4 mixtures.**  
(DOCX)

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## Author Contributions

Conceived and designed the experiments: JFJB SRM. Performed the experiments: MJM ARM EME ABF. Analyzed the data: MJM JFJB ARM EME SRM. Contributed reagents/materials/analysis tools: MJM ABF ARM EME. Wrote the paper: MJM SSK ARM EME SRM JFJB.

## References

1. Juárez-Montiel M, Ruiloba de León S, Chávez-Camarillo G, Hernández-Rodríguez C, Villa-Tanaca L. Huitlacoche (corn smut), caused by the phytopathogenic fungus *Ustilago maydis*, as a functional food. *Rev Iberoam Micol.* 2011; 28: 69–73. doi: [10.1016/j.riam.2011.01.001](https://doi.org/10.1016/j.riam.2011.01.001) PMID: [21352944](https://pubmed.ncbi.nlm.nih.gov/21352944/)
2. Ruiz-Herrera J, Martínez-Espinoza AD. The fungus *Ustilago maydis*: from the aztec cuisine to the research laboratory. *Int Microbiol.* 1998; 1: 149–58. PMID: [10943353](https://pubmed.ncbi.nlm.nih.gov/10943353/)
3. Valdez-Morales M, Barry K, Fahey GC Jr, Domínguez J, González de Mejía E, Valverde ME, et al. Effect of maize genotype, developmental stage, and cooking process on the nutraceutical potential of huitlacoche (*Ustilago maydis*). *Food Chem.* 2010; 119: 689–697.
4. Bölker M, Basse CW, Schirawski J. *Ustilago maydis* secondary metabolism-From genomics to biochemistry. *Fungal Genet Biol.* 2008; 45:S88–S93. doi: [10.1016/j.fgb.2008.05.007](https://doi.org/10.1016/j.fgb.2008.05.007) PMID: [18585066](https://pubmed.ncbi.nlm.nih.gov/18585066/)
5. Banuett F. Genetics of *Ustilago maydis*, a fungal pathogen that induces tumors in maize. *Annu Rev Genet.* 1995; 29: 179–208. PMID: [8825473](https://pubmed.ncbi.nlm.nih.gov/8825473/)
6. Banuett F, Herskowitz I. Discrete developmental stages during teliospore formation in the corn smut fungus, *Ustilago maydis*. *Development.* 1996; 122: 2965–2976. PMID: [8898211](https://pubmed.ncbi.nlm.nih.gov/8898211/)
7. Spellig T, Bölker M, Lottspeich F, Frnak RW, Kahmann R. Pheromones trigger filamentous growth in *Ustilago maydis*. *EMBO J.* 1994; 13: 1620–1627. PMID: [8157001](https://pubmed.ncbi.nlm.nih.gov/8157001/)
8. Gillenssen B, Bergemann J, Sandmann C, Schroeer B, Bolker M, Kahmann R. A two-component regulatory system for self/non-self recognition in *Ustilago maydis*. *Cell.* 1992; 68: 647–657. PMID: [1739973](https://pubmed.ncbi.nlm.nih.gov/1739973/)
9. Brachmann A, König J, Julius C, Feldbrügge M. A reverse genetic approach for generating gene replacement mutants in *Ustilago maydis*. *Mol Genet Genomics.* 2004; 272: 216–226. PMID: [15316769](https://pubmed.ncbi.nlm.nih.gov/15316769/)
10. Kämper J, Kahmann R, Bölker M, Ma LJ, Brefort T, Saville BJ, et al. Insights from the genome of the biotrophic fungal plant pathogen *Ustilago maydis*. *Nature.* 2006; 444: 97–101. PMID: [17080091](https://pubmed.ncbi.nlm.nih.gov/17080091/)
11. Terfrüchte M, Joehnk B, Fajardo-Somera R, Braus GH, Riquelme M, Schipper K, et al. Establishing a versatile golden gate cloning system for genetic engineering in fungi. *Fungal Genet Biol.* 2014; 62: 1–10. doi: [10.1016/j.fgb.2013.10.012](https://doi.org/10.1016/j.fgb.2013.10.012) PMID: [24211735](https://pubmed.ncbi.nlm.nih.gov/24211735/)
12. Feldbrügge M, Kellner R, Schipper K. The biotechnological use and potential of plant pathogenic smut fungi. *Appl Microbiol Biotechnol.* 2013; 97: 3253–3265. doi: [10.1007/s00253-013-4777-1](https://doi.org/10.1007/s00253-013-4777-1) PMID: [23455565](https://pubmed.ncbi.nlm.nih.gov/23455565/)



13. Rosales-Mendoza S, Salazar-González JA. Immunological aspects of using plant cells as delivery vehicles for oral vaccines. *Expert Rev Vaccines*. 2014; 13: 737–749. doi: [10.1586/14760584.2014.913483](https://doi.org/10.1586/14760584.2014.913483) PMID: [24766405](https://pubmed.ncbi.nlm.nih.gov/24766405/)
14. Shin MK, Yoo HS. Animal vaccines based on orally presented yeast recombinants. *Vaccine*. 2013; 31: 4287–4292. doi: [10.1016/j.vaccine.2013.07.029](https://doi.org/10.1016/j.vaccine.2013.07.029) PMID: [23891501](https://pubmed.ncbi.nlm.nih.gov/23891501/)
15. Wilkhu J, McNeil SE, Kirby DJ, Perrie Y. Formulation design considerations for oral vaccines. *Ther Deliv*. 2011; 2: 1141–1164. PMID: [22833911](https://pubmed.ncbi.nlm.nih.gov/22833911/)
16. Giddings G, Allison G, Brooks D, Carter A. Transgenic plants as factories for biopharmaceuticals. *Nat Biotechnol*. 2000; 18: 1151–1155. PMID: [11062432](https://pubmed.ncbi.nlm.nih.gov/11062432/)
17. Nevalainen H, Peterson R. Making recombinant proteins in filamentous fungi- are we expecting too much?. *Front Microbiol*. 2014; 5: 75. doi: [10.3389/fmicb.2014.00075](https://doi.org/10.3389/fmicb.2014.00075). eCollection. PMID: [24578701](https://pubmed.ncbi.nlm.nih.gov/24578701/)
18. Langridge W, Dénes B, Fodor I. Cholera toxin B subunit modulation of mucosal vaccines for infectious and autoimmune diseases. *Curr Opin Investig Drugs*. 2010; 11: 919–928. PMID: [20721834](https://pubmed.ncbi.nlm.nih.gov/20721834/)
19. George-Chandy A, Eriksson K, Lebens M, Nordström I, Schön E, Holmgren J. Cholera toxin B subunit as a carrier molecule promotes antigen presentation and increases CD40 and CD86 expression on antigen-presenting cells. *Infect Immun*. 2001; 69: 5716–5725. PMID: [11500448](https://pubmed.ncbi.nlm.nih.gov/11500448/)
20. Sun JB, Czerkinsky C, Holmgren J. Mucosally induced immunological tolerance, regulatory T cells and the adjuvant effect by cholera toxin B subunit. *Scand J Immunol*. 2010; 71: 1–11. doi: [10.1111/j.1365-3083.2009.02321.x](https://doi.org/10.1111/j.1365-3083.2009.02321.x) PMID: [20017804](https://pubmed.ncbi.nlm.nih.gov/20017804/)
21. Merritt EA, Sarfaty S, van den Akker F, L'Hoir C, Martial JA, Hol WG. Crystal structure of cholera toxin B-pentamer bound to receptor GM1 pentasaccharide. *Protein Sci*. 1994; 3: 166–175. PMID: [8003954](https://pubmed.ncbi.nlm.nih.gov/8003954/)
22. Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, et al. *Current protocols in molecular biology*. New York: Greene Publishing Associates and Wiley-Interscience; 1987.
23. Schmitt ME, Brown TA, Trumpower BL. A rapid and simple method for preparation of RNA from *Saccharomyces cerevisiae*. *Nucleic Acids Res*. 1990; 18: 3091. PMID: [2190191](https://pubmed.ncbi.nlm.nih.gov/2190191/)
24. Rodríguez-Kessler M, Baeza-Montañez L, García-Pedrajas MD, Tapia-Moreno A, Gold S, Jiménez-Bremont JF, et al. Isolation of UmRm75, a gene involved in dimorphism and virulence of *Ustilago maydis*. *Microbiol Res*. 2012; 167: 270–282. doi: [10.1016/j.micres.2011.10.007](https://doi.org/10.1016/j.micres.2011.10.007) PMID: [22154329](https://pubmed.ncbi.nlm.nih.gov/22154329/)
25. Hernández-Rodríguez AA, Ortega-Amaro MA, Delgado-Sánchez P, Salinas J, Jiménez-Bremont JF. *AtGRDP1* gene encoding a glycine-rich domain protein is involved in germination and responds to ABA signalling. *Plant Mol Biol Rep*. 2014; 32: 1187–1202. doi: [10.1007/s11105-014-0714-4](https://doi.org/10.1007/s11105-014-0714-4)
26. Holliday R. *Ustilago maydis*. In: King RC, editor. *Handbook of genetics*, vol. 1. New York: Plenum; 1974. pp. 575–595.
27. Franklin S, Ngo B, Efué E, Mayfield SP. Development of a GFP reporter gene for *Chlamydomonas reinhardtii* chloroplast. *Plant J*. 2002; 30:733–744. PMID: [12061904](https://pubmed.ncbi.nlm.nih.gov/12061904/)
28. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin Phenol Reagent. *J Biol Chem*. 1951; 193: 265–275. PMID: [14907713](https://pubmed.ncbi.nlm.nih.gov/14907713/)
29. Chikwamba RL, Cunnick J, Hathaway D, McMurray J, Mason H, Wang K. Functional antigen in a practical crop: LT-B producing maize protects mice against *Escherichia coli* heat labile enterotoxin (LT) and cholera toxin (CT). *Transgenic Res*. 2002; 11: 479–493. PMID: [12437079](https://pubmed.ncbi.nlm.nih.gov/12437079/)
30. Rosales-Mendoza S, Soria-Guerra RE, López-Revilla R, Moreno-Fierros L, Alpuche-Solís AG. Ingestion of transgenic carrots expressing the *Escherichia coli* heat-labile enterotoxin B subunit protects mice against cholera toxin challenge. *Plant Cell Rep*. 2008; 27: 79–84. PMID: [17874110](https://pubmed.ncbi.nlm.nih.gov/17874110/)
31. Richardson SH, Giles JC, Kruger KS. Sealed adult mice: new model for enterotoxin evaluation. *Infect Immun*. 1984; 43: 482–486. PMID: [6363287](https://pubmed.ncbi.nlm.nih.gov/6363287/)
32. Spellig T, Bottin A, Kahmann R. Green fluorescent protein (GFP) as new vital marker in the phytopathogenic fungus *Ustilago maydis*. *Mol Gen Genet*. 1996; 252: 503–509. PMID: [8914511](https://pubmed.ncbi.nlm.nih.gov/8914511/)
33. Soria-Guerra RE, Moreno-Fierros L, Rosales-Mendoza S. Two decades of plant-based candidate vaccines: a review of the chimeric protein approaches. *Plant Cell Rep*. 2011; 30: 1367–1382. doi: [10.1007/s00299-011-1065-3](https://doi.org/10.1007/s00299-011-1065-3) PMID: [21505834](https://pubmed.ncbi.nlm.nih.gov/21505834/)
34. Rosales-Mendoza S, Paz-Maldonado LM, Soria-Guerra RE. *Chlamydomonas reinhardtii* as a viable platform for the production of recombinant proteins: current status and perspectives. *Plant Cell Rep*. 2012; 31: 479–494. doi: [10.1007/s00299-011-1186-8](https://doi.org/10.1007/s00299-011-1186-8) PMID: [22080228](https://pubmed.ncbi.nlm.nih.gov/22080228/)
35. Hamorsky KT, Kouokam JC, Bennett LJ, Baldauf KJ, Kajiura H, Fujiyama K. Rapid and scalable plant-based production of a cholera toxin B subunit variant to aid in mass vaccination against Cholera outbreaks. *PLoS Negl Trop Dis*. 2013; 7: e2046. doi: [10.1371/journal.pntd.0002046](https://doi.org/10.1371/journal.pntd.0002046) PMID: [23505583](https://pubmed.ncbi.nlm.nih.gov/23505583/)
36. Harakuni T, Sugawa H, Komesu A, Tadano M, Arakawa T. Heteropentameric cholera toxin B subunit chimeric molecules genetically fused to a vaccine antigen induce systemic and mucosal immune

- responses: a potential new strategy to target recombinant vaccine antigens to mucosal immune systems. *Infect Immun*. 2005; 73: 5654–5665.
37. Payero JO, Melvin SR, Irmak S, Tarkalson D. Yield response of corn to deficit irrigation in a semiarid climate. *Agric Water Manage*. 2006; 84: 101–112.
  38. Twyman RM, Stoger E, Schillberg S, Christou P, Fischer R. Molecular farming in plants: host systems and expression technology. *Trends Biotechnol*. 2003; 21: 570–578. PMID: [14624867](#)
  39. Ward OP. Production of recombinant proteins by filamentous fungi. *Biotechnol Adv*. 2012; 30: 1119–1139. doi: [10.1016/j.biotechadv.2011.09.012](#) PMID: [21968147](#)
  40. Matsumoto Y, Suzuki S, Nozoye T, Yamakawa T, Takashima Y, Arakawa T, et al. Oral immunogenicity and protective efficacy in mice of transgenic rice plants producing a vaccine candidate antigen (As16) of *Ascaris suum* fused with cholera toxin B subunit. *Transgenic Res*. 2009; 18: 185–192. doi: [10.1007/s11248-008-9205-4](#) PMID: [18763047](#)
  41. Nozoye T, Takaiwa F, Tsuji N, Yamakawa T, Arakawa T, Hayashi Y, et al. Production of *Ascaris suum* As14 protein and its fusion protein with cholera toxin B subunit in rice seeds. *J Vet Med Sci*. 2009; 71: 995–1000. PMID: [19652493](#)
  42. Karaman S, Cunnick J, Wang K. Expression of the cholera toxin B subunit (CT-B) in maize seeds and a combined mucosal treatment against cholera and traveler's diarrhea. *Plant Cell Rep*. 2012; 31: 527–537. doi: [10.1007/s00299-011-1146-3](#) PMID: [21938449](#)
  43. Daniell H, Singh ND, Mason H, Streatfield SJ. Plant-made vaccine antigens and biopharmaceuticals. *Trends Plant Sci*. 2009; 14: 669–679. doi: [10.1016/j.tplants.2009.09.009](#) PMID: [19836291](#)
  44. Miyata T, Harakuni T, Taira T, Matsuzaki G, Arakawa T. Merozoite surface protein-1 of *Plasmodium yoelii* fused via an oligosaccharide moiety of cholera toxin B subunit glycoprotein expressed in yeast induced protective immunity against lethal malaria infection in mice. *Vaccine*. 2012; 30: 948–958. doi: [10.1016/j.vaccine.2011.11.059](#) PMID: [22119928](#)
  45. Wang LX, Lomino JV. Emerging technologies for making glycan-defined glycoproteins. *ACS Chem Biol*. 2012; 7: 110–122. doi: [10.1021/cb200429n](#) PMID: [22141574](#)
  46. Bosch D, Schots A. Plant glycans: friend or foe in vaccine development?. *Expert Rev Vaccines*. 2010; 9: 835–842. doi: [10.1586/erv.10.83](#) PMID: [20673008](#)
  47. Stock J, Sarkari P, Kreibich S, Brefort T, Feldbrügge M, Schipper K. Applying unconventional secretion of the endochitinase CTS1 to export heterologous proteins in *Ustilago maydis*. *J Biotechnol*. 2012; 161: 80–91. doi: [10.1016/j.jbiotec.2012.03.004](#) PMID: [22446315](#)
  48. Fernández-Alvarez A, Elías-Villalobos A, Ibeas JI. Protein glycosylation in the phytopathogen *Ustilago maydis*: From core oligosaccharide synthesis to the ER glycoprotein quality control system, a genomic analysis. *Fungal Genet Biol*. 2010; 47: 727–735. doi: [10.1016/j.fgb.2010.06.004](#) PMID: [20554055](#)
  49. Maskery B, DeRoeck D, Levin A, Kim YE, Wierzba TF, Clemens JD. Strategy, demand, management, and costs of an international cholera vaccine stockpile. *J Infect Dis*. 2013; 208 Suppl 1: S15–22. doi: [10.1093/infdis/jit233](#) PMID: [24101640](#)
  50. Basset C, Thiam F, Martino CD, Holton J, Clements JD, Kohli E. Cholera-like enterotoxins and Regulatory T cells. *Toxins (Basel)*. 2010; 2: 1774–1795. doi: [10.3390/toxins2071774](#)