Plant Based Enhancement of Cholera Toxin B Subunit Expression for Oral Immunisation Strategies

Saravanan Ravindran(1)*, Dhachinamoorthi Duraiswamy(2), Jalaiah Marri(3), Sasidhar Bhimana(4) , Malayadri Talluri(5)

1. Professor, QIS College of pharmacy, Vengamukkapalem, Ongole, A.P, India – 523272.
E-mail: rssrmcp@gmail.com; Mobile: 91-9700784826.
2. Professor, QIS College of pharmacy, Vengamukkapalem, Ongole, A.P, India - 523272
3. Associate Professor, QIS College of pharmacy, Ongole, Vengamukkapalem, A.P, India - 523272
4. Assistant Professor, QIS College of pharmacy, Ongole, Vengamukkapalem, A.P, India – 523272
5. Assistant Professor, QIS College of pharmacy, Ongole, Vengamukkapalem, A.P, India – 523272

ABSTRACT:
Optimized expression of the Cholera Toxin B subunit (CT-B) in edible plants is a green, cheap and scalable idea for the next generation of oral vaccines. CT-B is the harmless part of the cholera toxin that maintains strong binding to GM1-ganglioside receptors on intestinal epithelial cells, allowing easy mucosal uptake and subsequent immune activation. Nevertheless, the establishment of a stable, and high-level of CT-B accumulation in plant tissues is still the major bottleneck of this technology. The present research is devoted to the investigation of different approaches in the production of CT-B in the edible plants i.e. codon optimisation, incorporation of plant-preferred regulatory sequences, fusion of transit peptide to targeted subcellular localisation, and the use of expression systems that are capable of high protein yields. Lettuce, tomato, and banana as edible plants are highly desirable because of their raw consumability, and they are easy to cultivate and propagate on a large scale. Alteration of gene constructs and promoter selection for CT-B expression leads to higher antigen production while still maintaining the proper folding of the protein and assembly of the pentamer, which are critical for biological activity. When the CT-B is targeted to the endoplasmic reticulum or chloroplast usually the protein is more stable and accumulates more, thus the immunologically relevant oral doses are more achievable. Also, CT-B from plants is structurally identical to the native one and hence it can bind mucosal surfaces and induce systemic as well as local immune responses. Genetically modified edible plants with CT-B will surely have an immense potential to reduce production capital, avoid the need for cold storage, and provide a comprehensive way of immunization through the oral route focused on cholera prone regions. The next steps include ongoing refinement of the expression methods, antigen storage stability, and immune response studies that are necessary for making CT-B from edible plants a practical oral vaccine candidate.

Keywords: Cholera Toxin B Subunit, Edible Plants, Oral Immunisation, Plant-Based Vaccines, Antigen Expression, Mucosal Immunity

INTRODUCTION

Cholera ranks as one of the most tenacious and damaging intestinal diseases of mankind. It is a disease that is essentially characterized by the severe dehydration resulting from the diarrheal disease caused by the bacteria Vibrio cholerae, mainly the O1 and O139 serogroups. For around two centuries, the disease has been a major concern of public health worldwide, with pandemics being reported from as early as the 19th century. The first documented pandemic was in 1817 and the place of origin was the Ganges Delta. From there it spread rapidly to Asia, the Middle East, and Eastern Europe. After that, the epidemics kept returning in the 19th and 20th centuries respectively, demonstrating how fast the causative agent can be transmitted in densely populated places with contaminated water and poor sanitation. The seventh pandemic that is still continuing and is mostly associated with the El Tor biotype has been affecting millions of people, primarily in areas where health systems are weak, there is limited access to clean water, and the humanitarian crises are ongoing. Although drinking water has become safer and there are better hygiene practices, cholera is still a problem in some parts of Africa, South Asia, and Latin America [1]. The outbreaks that keep occurring in these areas reveal the limitations of the current control measures that are in place. The fact that cholera outbreaks have been happening repeatedly over the years has always been interpreted as a signal that there is a continuous demand for effective, affordable, and easy-to-use vaccines. Traditional injectable vaccines have hardly been effective in disease control since they are associated with logistical difficulties and the fact that they bring about only a modest mucosal immunity, which is very important for protection against pathogens of the intestines [2]. Presently, oral cholera vaccines (OCVs) like whole-cell inactivated or weakened formulations have now come to terms by enhancing both systemic and mucosal immune responses. There are issues with the storage of oral vaccines, and their shelf life depends upon the cold chain requirements, along with the cost of production limits the application in large-scale circumstances. These difficulties have made people think about other vaccine platforms that can facilitate mass vaccination especially in resource-poor areas. As a result, the plant-based vaccines have attracted attention as they have the capability of producing antigens in edible tissues which enables easy oral delivery without complicated processing [3].

The cholera toxin B subunit (CT-B), which represents the non-toxic part of the cholera toxin complex, is the antigen that has turned out to be the most significant one in the development of plant-based oral vaccines. In nature, CT-B associates into a very stable pentameric structure capable of binding GM1-ganglioside receptors on the surface of intestinal epithelial cells with very high affinity thus, opening the way for facile transport across the mucosal surface [4]. This receptor-binding feature is the main driver not only of antigen delivery since it also allows CT-B to behave as an immunological adjuvant, thus, favoring both local and systemic immune responses. Since CT-B is a component in licensed OCVs, its safety and immunogenic relevance have already been confirmed, which makes it a perfect candidate for the expression systems based on plants. Edible crops such as lettuce, tomato, potato, rice, and banana are the most valuable sources of immunogenic proteins which can be directly delivered by consumption thereby, purification, sterile injection equipment, and the services of a trained person are no longer necessary. The idea of generating CT-B in edible plants comes with several pros but at the same time, obtaining a high and stable expression of the antigen remains a daunting technical challenge [5]. In most cases, early experiments reported the low accumulation of protein as a result of weak promoters, non-optimized codon usage, protein folding inefficiency, and vulnerability to proteolytic degradation of the plant tissues. Results from experiments of this nature emphasized the necessity to optimise gene constructs and expression strategies so as to elevate antigen levels. More to this is codon optimization which is specific to the plant since the promoter to be used should be really strong and the regulatory sequences for mRNA stability should be included. Also, targeting is one of the important optimization factors [6]. Transferring CT-B to the endoplasmic reticulum, chloroplast, or protein storage vacuoles can be supported for protein folding, pentamer assembly, and storage, respectively, thereby leading to an increase of the antigen concentration in the edible portion of the plant.

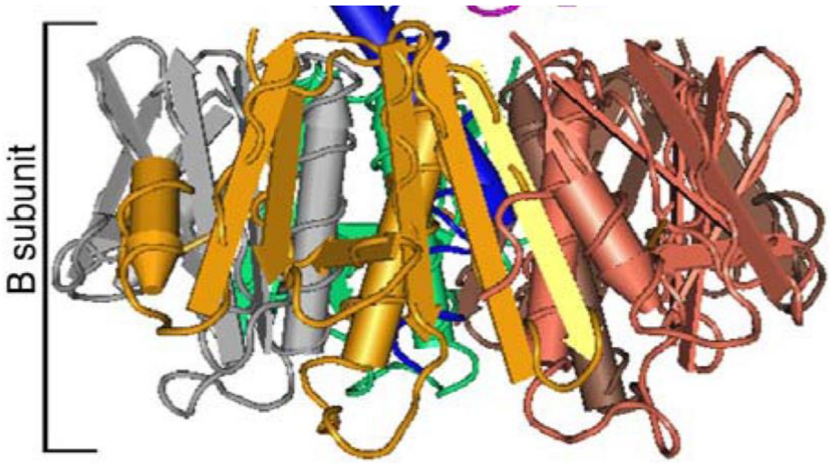

Fig 1. Three-dimensional structure of pentameric CT -B subunit [7]

Moreover, Plant biotechnology innovations more than ever offer a wide array of options for the expression of CT-B. Hence, transient expression systems which make use of plant viral vectors help in the rapid and high-level production of antigens whereas, stable transgenic line studies serve the long-term generation and scalability purposes. Since maternal inheritance is one of the advantages of chloroplast transformation, it alleviates concerns about gene flow while it is also possible to produce extremely high antigen yields due to the polyploidy nature of chloroplast genomes [8]. Additionally thereby, the existence of fast-growing edible plant species with minimum cultivation requirements would be valuable for community-based production models and, especially, during emergency outbreak responses. The use of plant-derived CT-B has been extended to numerous other applications beyond immunisation against cholera alone. Since CT-B exhibits potent immunomodulatory properties to mucosal, plant-based form of CT-B can have the potential to become a component of multi-antigen oral vaccines, carrier molecules, or adjuvants for other enteric diseases. Besides, plant-based vaccine production is in line with the strategies that are intended to reduce the dependence on the centralised manufacturing facilities. Plant growing can be tailored to the agricultural environments, community-level greenhouses, or controlled hydroponic systems, etc., thus providing a mode of vaccine production that can be decentralised and flexible where there are hardly any conventional pharmaceutical facilities [9].

In spite of the promising developments, the edible plant-derived CT-B vaccines in clinical settings are still far from being realized due to the existing problems that need to be solved first. Expression variability-, production consistency-, unintentional exposure risks-, regulation-, and acceptance of genetically modified plant products by the public are still the aspects to be considered. In addition to that, the longevity of CT-B in situations leading up to its administration should also be paid attention to, for example, the temperature, humidity, and conditions of the post-harvest period may all have an impact on the antigen integrity. However, these limitations are progressively being resolved by means of controlled cultivation, encapsulation, strict biosafety measures, and better communication of the benefits and risks of plant-based vaccines [10]. These vaccines are greatly validated through genome editing, synthetic biology, and next-gen systems. CRISPR-based precision editing could also be employed to optimize transgene integration accuracy, which meets regulatory guidelines. Antigen production may also be enhanced through the use of synthetic promoters and metabolic engineering tools. The use of bioencapsulation methods such as the protection of plant cell walls and freeze-drying can thus not only stabilize the antigen but also enable a distribution that is free of refrigeration. While scientists are still working through the technical and regulatory challenges of this approach, edible plant-derived CT-B vaccines may become inexpensive, widely implemented, and scalable instruments to combat cholera, especially in regions where vaccination is constrained by economic and logistical factors [11].

SUSTAINABLE PLANT PLATFORMS FOR THE PRODUCTION OF VACCINE ANTIGEN

Edible plants are now one of the most attractive means for the creation of vaccines that can be taken orally. They offer a viable alternative to the traditional ways of vaccine production. The idea of producing recombinant antigens in the tissue of plants that can be eaten opens up a completely different way of immunisation- the first option is local people in remote areas who suffer from lack of cold chain storage, sterile injection equipment and trained personnel [12]. With the advances in plant molecular biology for the last 30 years, it has become possible to genetically modify edible crops that include lettuce, tomato, potato, banana, carrot, maize, and rice to express clinically relevant antigens such as cholera toxin B subunit (CT-B), hepatitis B surface antigen, norovirus capsid protein and rotavirus VP6. These organisms are equipped with the machinery to produce biologics that can be taken up by the mucosa without further processing, thus, they can be considered the best delivery system for oral vaccines [13]. The use of edible plant platforms provides numerous benefits that make them perfect candidates for antigen production. Their safety, non-pathogenic nature, and global acceptance as food, give them the advantage that no extensive purification steps are required, and they can be consumed directly. Moreover, antigens that are enclosed in plant cells are only partly exposed to the degrading enzymes in the gastrointestinal tract; hence, the chance of the delivery to the intestinal mucosa is also increased. Besides, cultivation of plants is less demanding in terms of the facilities than microbial or mammalian cell-based systems and is therefore doable at the local or regional level in small-scale productions [14]. The versatility of plant agriculture also slows the process of rapid scale-up during a pandemic; hence edible plant-based vaccines are crisis management tools, especially for quick response to emergency situations [15].

Among edible crops, leafy vegetables such as lettuce have received particular interest because of their fast growth cycles, high biomass, and adaptability to open-field and controlled-environment cultivation. Lettuce is eaten raw, so temperature-sensitive antigens like CT-B are preserved without the denaturation that occurs during cooking. Other model systems are tomato and potato. Tomatoes are juicy and delicious while potatoes possess long shelf life and are easy bulk transport [16]. Rice and maize have been investigated for directed expression of antigens in seeds because seeds represent natural, stabilizing matrices, which can preserve immunogenic proteins for months without refrigeration. This seed-based expression approach has unique advantages for developing thermostable oral vaccines for global immunisation campaigns in developing countries with minimal resources. However,the stable, high-level, and long-term accumulation of vaccine antigens in edible tissues is very challenging. Different plant-specific post-translational modifications may change the folding of one antigen or its immunogenicity, and proteolytic degradation occurring in plant cells may limit the amount of one antigen. Currently, several approaches have been developed to overcome these obstacles [17]. These approaches include codon optimization, fusion of antigens with carrier peptides, and subcellular targeting. Antigens in the endoplasmic reticulum can obtain the proper folding and disulfide bond formation, while protein storage vacuoles or chloroplasts can help protect protein from degradation. Promoter engineering has also enabled a remarkable increase in transcriptional efficiency achieved by strong constitutive promoters (such as CaMV 35S) or tissue-specific promoters which are highly expressed in edible tissues [18].

Inducing mucosal immunity is the most important benefit of edible plant vaccine platforms. It is well known that most pathogens, e.g., Vibrio cholerae, enter through mucosal surfaces, hence the stimulation of secretory IgA responses is the central mechanism of protection. Edible plants expressing CT-B have been reported as good inducers of mucosal immunity, the reason is that the antigen binds naturally to GM1-ganglioside receptors on intestinal epithelial cells [19]. The receptor-mediated uptake leads to enhanced antigen presentation to mucosal-associated lymphoid tissues, thus the immune response generated is systemic as well as mucosal without occurrence from adjuvants or injections. Plant-derived vaccines administered through the oral route are still being researched, with the early translational stage being the furthest reached, but they can be used far beyond that of conventional immunisation [20]. A few possibilities come to mind: first, combination vaccines manufacturing; then, edible boosters; lastly, the paediatric or elderly populations-targeted antigen formulations. Utilising them in community-level production models is a great way to show their decentralised healthcare systems' potential. With technological breakthroughs in genome-editing, regulatory harmonisation, and advancing in antigen stability, edible plants will be the ones who revolutionise the next generation of low-cost oral vaccines accessible to all people worldwide [21].

STRUCTURE, FUNCTION, AND IMMUNOLOGICAL SIGNIFICANCE OF CT - B ANTIGEN

The cholera toxin B subunit (CT-B) is the part of the cholera toxin complex that is very important and is the main factor that connects host–pathogen at the intestinal mucosa surface. When we look at the protein structurally, CT-B is composed of five identical units (homopentamer), and each unit weighs around 11.6 kDa [22].These five identical monomers unite themselves into a perfect ring-like format that shows the highest affinity binding area for GM1-ganglioside receptors that are on the surface of epithelial cells. The immunological characteristics as well as receptor binding and biological activity of pentameric CT-B make it one of the most immunogenic antigens used in oral vaccines. The tightly packed and very precisely arranged pentameric molecule is also the major factor in its stability in the human body, thus it is possible for it to resist the harsh environment of the gut if given orally [23].

The very first and main biological function of the CT-B is to attach to GM1 receptors on the surface of intestinal epithelial cells. As a result of this, the whole toxin complex through receptor-mediated endocytosis is internalized into host cells. It is worth noting that CT-B is not harmful itself since it does not possess any enzymatic activity like the one of the A subunit which is responsible for increasing cyclic AMP and chloride secretion [24]. Nevertheless, the use of CT-B as a receptor-binding agent greatly facilitates antigen presentation and mucosal uptake thus, making it the most suitable component in oral vaccine production. Acting as a natural ligand for GM1, CT-B thus ensures the very efficient delivery of antigens to the gut-associated lymphoid tissue (GALT) which is the place where immune responses take off. a purely binding unit, CT-B has certain immunomodulatory effects that are inherent in the molecule [25]. One of the features is that it can strongly induce IgA (sIgA) production in the intestinal mucosa thus raising the mucosal immunity potential against enteropathogens. sIgA blocks the process of pathogen binding and dissemination, thus, represents the first line of defense in the mucosal immune system [26]. Besides, CT-B also triggers systemic immune responses, thus, IgG production is enhanced and cellular immune pathways are activated upon interaction with antigen-presenting cells such as dendritic cells and macrophages. This ability to elicit both mucosal and systemic immune responses is the main reason why it is the best vaccine antigen.

One more major immunological characteristic of CT-B is its adjuvant-like behavior. In addition to recognizing itself, CT-B also makes the immune system aware of antigens that are given with it, thus it becomes a very effective mucosal adjuvant [27]. In many experimental models, the scientists have observed that CT-B can change cytokine responses and help the Th2 immune system pathway. Due to the fact that CT-B when connected with other antigens can deliver them more efficiently to mucosa and increase the general immunogenicity, it is very likely that multivalent oral vaccine platforms will utilize this concept to produce more vaccines. Moreover, the structural resilience of CT-B is one of the factors why it can be used for the production of vaccines in plants [28]. A stable pentameric form as well as the correct folding of CT-B can be done in the plant cells, especially if the protein is targeted to the subcellular organelles like the endoplasmic reticulum where the formation of the disulfide bond is a requirement for the proper assembly [29]. Also, the stability of CT-B makes it possible to store vaccines obtained from plants as well as deliver them to different vaccines , thus they can be very easily handled in the setting of insufficient resources [30]. The combined structural configuration, receptor-binding properties, immunomodulatory functions, and natural mucosal targeting ability of CT-B are the main reasons why it is one of the most effectively and extensively studied antigens for oral vaccine development. In addition to strong mucosal immunogenicity, safety profile of CT-B, and compatibility with plant expression systems the interest in CT-B as a component of oral next-generation vaccines targeting cholera and potentially other enteric infections does not wane.

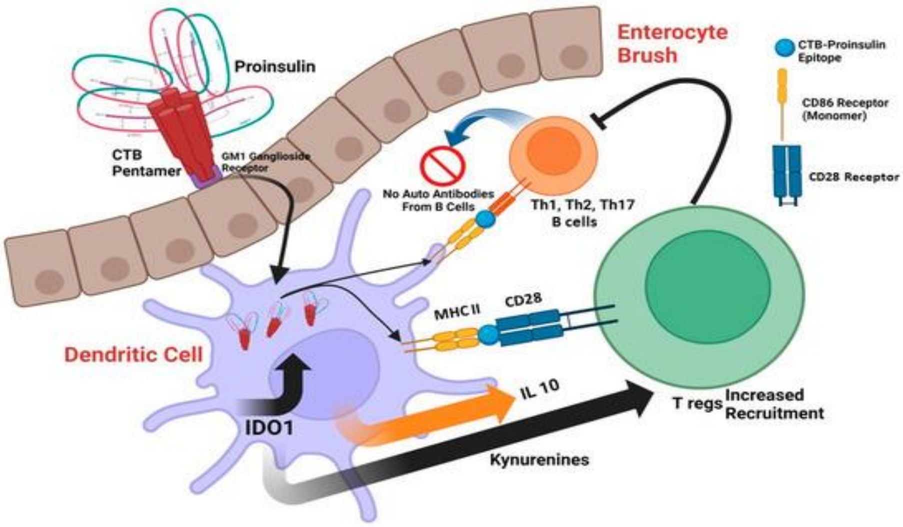

Fig 2. Proposed mechanism for the CTB-INS edible vaccine. [31]

MOLECULAR TECHNIQUES TO ENHANCE PLANT CT - B EXPRESSION

One of the central requirements for the development of edible vaccines and oral immunisation platforms is the optimisation of the expression of the cholera toxin B subunit (CT-B) in plants. CT-B is a very sensitive antigen from the structural point of view and it has to fold properly and furthermore, form a pentamer in order to have the capacity to bind its receptor and be immunologically active. Even though plant systems have advantages that are not offered by other systems in the production of recombinant proteins, they still pose challenges such as fluctuation in expression levels, proteolytic degradation, and difference in post-translational processing. In the last 30 years, a variety of molecular strategies have been proposed to bolster the production of CT-B in terms of its synthesis, stability, folding, and accumulation in the plant tissues. These strategies involve gene design, choice of regulatory elements, subcellular localisation, transformation systems as well as stability technologies and protein stability.

1. CODON OPTIMIZATION

Among the various strategies to enhance the expression of CT-B in edible plants, codon optimization stands out as one of the most significant ones. Since the codon usage of bacteria (the original source of CT-B) and plants is different, the sequences of bacteria that have not been modified are often translated with low efficiency. Modification of bacterial sequences through plant-preferred codons increases the efficiency of translation and at the same time provides the correct folding since no ribosomal pausing occurs. This step is extremely important for CT-B as the subunit has to be structurally very precise in order to bind GM1-ganglioside receptors. Besides codon optimisation, removal of RNA destabilising sequences and optimising GC content are often done simultaneously to stabilise mRNA [32].

2. **PROMOTOR ENGINEERING**

Promoter engineering has been one of the crucial factors that has largely influenced the yield of CT-B. Top constitutive promoters like Cauliflower Mosaic Virus 35S (CaMV 35S), Ubiquitin promoters, or elevated synthetic promoters are capable of sweeping a large variety of plant tissues with high mRNA levels. Besides that, tissue-specific promoters gain the additional advantages; for instance, fruit-specific promoters in tomatoes, tuber-specific promoters in potatoes, and seed-specific promoters in rice or maize that not only promote accumulation in the edible parts but also minimize the unnecessary metabolic load of non-targeted tissues. There is also the possibility of using inducible promoters for the purpose of switching-on CT-B expression only upon a certain chemical or environmental stimulus and thus, by this way, avoid toxicity and metabolic burden during the growth of the plant [33].

3. **ENHANCER SEQUENCES AND UNTRANSULATED REGIONS (UTRs)**

Moreover, these elements do not only initiate expression but, in fact, they can go on rounding-out the gene with enhancer regions and untranslated regions (UTRs) that can stabilise transcripts and facilitate ribosome binding. They can actually shoot up the rate of expression considerably by making a big difference in the flow from the mRNA towards production. On the other hand, similarly, optimised 3′ UTRs and polyadenylation signals not only ease the translation process but they also provide the mRNAs with a longer life span [34].

4. SUBCELLULAR TARGETING

Among all, the subcellular targeting is undoubtedly the one that has the loudest voice when it comes to surface strategies of increasing not only CT-B stability but also pentamer formation assurance. The CT-B is a molecule whose chains are held by several disulfide bonds, and the correct folding is very important for the production of antibodies. The use of signal peptides along with the C-terminal ER retention sequence (KDEL) to direct CT-B to the endoplasmic reticulum (ER) not only facilitates the correct folding, but it also stimulates the formation of disulfide bonds and limits proteolysis. Retention in the ER is, in fact, one of the most successful ways of CT-B accumulation in plants as many experiments have shown the expression level to be much higher than that of the cytosol [35].

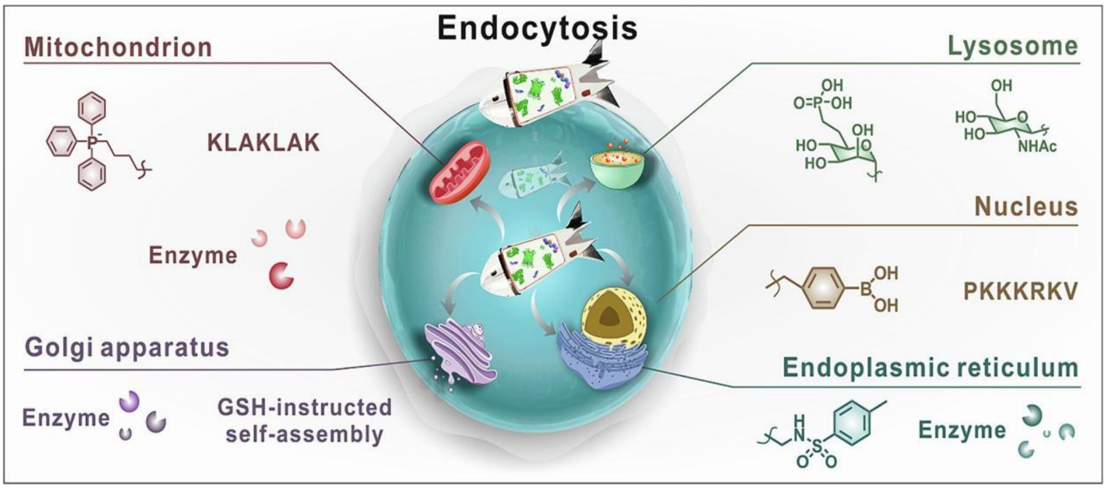

Fig 3. Subcellular targeting strategies [36]

5. CHLOROPLAST TRANSFORMATION

Besides that, chloroplast transformation can be counted as one more very efficient way. Chloroplasts are very capable of very high-level accumulation of recombinant proteins due to the high number of their genomes and the very powerful transcriptional machinery. In most cases, CT-B from chloroplasts is correctly folded and the pentamer is formed, and hence, there's no gene flow via pollen in most edible crops, which, in turn, increases biosafety. Besides that, chloroplasts are not glycosylated to a great extent, which is an advantage because CT-B is a non-glycosylated protein. Quite often, expression in chloroplasts leads to immunogen levels that are several times higher than the oral immunogenicity threshold [37].

6. FUSION TAGS AND CARRIER PROTEINS

For some uses, fusion tags and carrier proteins are utilized to promote CT-B accumulation. If fused to thermostable proteins or elastin-like polypeptides, the stability of the protein can be enhanced, and the process of the protein isolation after the vaccine production can be made simpler when the purification-based vaccine formats are used. The fusion of a signal peptide to a protein body or storage vacuole that targets the peptide fragment can protect CT-B from being digested by proteases and at the same time, it can support seed or tuber storage for a long period [38].

7. TRANSFORMATION SYSTEM

The decision to use which transformation system is yet another vital point. A stable nuclear transformation will allow production over several plant generations, while transient expression systems—like agroinfiltration with viral vectors—can be used to quickly obtain high levels of CT-B for a research lab or a sudden outbreak of a disease scenario. By using replicating RNA intermediates, viral vectors greatly increase transcriptional activity which is why they yield higher protein levels in a very short time [39]

8. GYLCOENGINEERING AND MOLECULAR CHAPERONES

Besides that, glycoengineering and molecular chaperones also help to raise the level of CT-B expression. Coexpression of some chaperones like protein disulfide isomerase (PDI) or binding protein (BiP) in the case of CT-B which does not need glycosylation, folding efficiency is improved and misassembly is lowered. The plant's endogenous protease activity can be modulated or the expression environment can be changed to further enhance the stability [40]

9. GENOME EDITING

At the end of the day, genome editing tools like CRISPR/Cas offer the possibility of very accurate integration of CT-B genes into genomic "safe harbours" which have high transcriptional activity. This way gene silencing is reduced and the expression is stable even in different generations. There are also more gadgets for the doctor's kit offered by synthetic biology like modular cloning systems and engineered metabolic pathways that could be used for expression fine-tuning and protein quality improvement [41].

Table 1. Cholera toxin B subunit (CT-B) expression in plants peer-reviewed studies [42-50]

S.No	Research Institute	Country	Plant Platform	Optimisation Mechanism	Reported CT-B Accumulation & Assembly	Immunogenicity
1	Loma Linda University, 1998	USA	Potato (tuber + leaf)	Nuclear transformation; CT-B gene fused to ER-retention signal (SEKDEL) under mannopine promoter	~ 0.3% total soluble protein (tuber & leaf); CT-B assembled into oligomers/pentamers.	Oral immunisation in mice led to serum + intestinal (mucosal) CT-B–specific antibodies; Vero cell neutralization of cholera toxin; ~60% reduction in intestinal fluid accumulation upon challenge.
2	University of Delhi South	India	Tomato (fruit + leaves)	Agrobacterium-mediated nuclear	Up to ~ 0.04% total soluble protein in fruit;	No animal immunisation reported in that

	Campus / Centre for Biochemical Technology, 2002			transformation; CT-B gene with ER-retention (SEKDEL), driven by CaMV 35S promoter.	~0.02% in leaves. Plantderived CT-B bound to GM1 receptor (GM1-ELISA), indicating pentamer formation. Demonstrated correct folding and receptor binding capacity	study.
3	Tokyo University (Rice research group), 2008	Japan	Rice (seed/endosperm)	Synthetic CT-B gene codon-optimised for plants; driven by a seed-specific promoter (wheat Bx17 HMW promoter + rice act1 intron), via biolistic transformation.	CT-B accumulated up to ~2.1% of total soluble protein in rice endosperm; plant-derived CT-B bound strongly to GM1-ganglioside, indicating correct pentamer assembly	Functional receptor-binding that supports feasibility of rice-based edible vaccine. No in vivo immunisation data in that report.
4	Combined CT-B + enterotoxin B (LT-B) study, transgenic rice — SpringerPlus, 2015	Asian countries	Rice (seed)	Transgenic expression of CT-B and LTB under suitable promoters; stable transformation in rice.	CT-B and LTB detected in rice seed protein extracts; CT-B detectable by ganglioside-dependent ELISA (GM1-binding), indicating pentameric / functional form.	In mice: oral immunisation with rice-derived CT-B (and/or LTB) elicited systemic IgG and mucosal IgA responses
5	University	USA	Tobacco	Chloroplas	CT-B	Chloroplast-

	of Central Florida (plastid engineering group), 2001		(leaf; non-food model)	t (plastid) genome transformation with unmodified CT-B gene under plastid promoter, enabling high-copy plastid expression	accumulation up to ~ 4.1% of total soluble leaf protein; CT-B assembled into functional oligomers/pentamers; antigenically identical to native bacterial CT-B.	produced CT-B retained GM1-ganglioside binding capacity — demonstrating correct folding, disulfide bond formation, and functional assembly.
6	Transient-expression study in Nicotiana benthamiana — viral-vector mediated expression, 2013	Sweden	N. benthamiana (leaf biomass; non-edible)	Use of plant-virus vector, secretory signal + ER-retention, aglycosylated CTB variant (pCTB) to improve yield and avoid plant-specific glycosylation	Yield > 1 g CT-B per kg fresh leaf material (very high), CT-B assembled into homo-pentamers; conformational stability and GM1-binding comparable to bacterial CT-B.	Oral immunisation in mice: induced robust intestinal (mucosal) CT-B–specific antibodies that persisted for at least 6 months; antibodies neutralized cholera holotoxin in vitro.
7	Study of maize seed–based CT-B expression, 2009	USA	Maize (seed) Synthetic	CT-B under control of seed-specific γ-zein promoter; stable transformation.	In T1 generation, CT-B at ~ 0.0014% of total soluble protein (TASP); in T2 generation increased to ~ 0.0197% TASP.	Oral immunisation of mice with maize-derived CT-B induced anti-CTB IgG (serum) and IgA (fecal), and protected against cholera holotoxin challenge. Combined CT-B + LT-B gave enhanced responses.

8	Fruit-specific expression in tomato under fruit-specific promoter (E8) — tomato CT-B edible vaccine study, 2007	Asian countries	Tomato	Agrobacterium-mediated transformation; CT-B under control of tomato fruit-specific E8 promoter for fruit-restricted expression.	CT-B accumulation in ripening tomato fruit: up to ~ 0.081% of total soluble protein. GM1-binding (pentameric CT-B) confirmed.	Oral feeding of ripe transgenic tomato fruit to mice induced both systemic (IgG) and mucosal (IgA) CT-B–specific antibodies.
9	Global Campus, College of Life Sciences, Kyung Hee University	South Korea	Rice seed	Transgenic nuclear transformation; CT-B + Heat-labile enterotoxin B subunit (LT-B) co-expression under seed-promoter (globulin promoter)	CT-B detectable in seed protein; GM1-ganglioside binding confirmed by G_{m1}-ELISA.	Oral or intraperitoneal immunisation in mice: serum IgG (i.p.) and, for oral CTB/LTB, toxin-neutralizing activity; elevated IgG & IgA in orally immunised mice.

EVALUATION OF CT-B ASSEMBLY, STABILITY, AND BIOACTIVITY IN PLANT TISSUES

1. **Structural Requirements for CT-B Pentamer Formation in Plant Cells**
   Production of plant-based cholera toxin B subunit (CT-B) oral vaccine candidate must first of all ensure correct folding and assembly of the pentamer, retention of GM1-ganglioside receptor binding activity and the stability of the antigen during plant growth, harvest, storage and processing. These factors together determine whether the antigen expressed in plant tissue still retains the structural and functional attributes required to elicit a protective mucosal immune response. Therefore, understanding CT-B assembly, stability, and bioactivity in plants is an essential of any plant-based vaccine development strategy [51]

2. **Verification of Pentamer Assembly Using Biochemical and Biophysical Assays**
CT-B is a pentamer of 5 identical subunits, which binds to a central pore and the antigen can bind to GM1 receptors on intestinal epithelial cells surfaces. Pentamerization is essential because GM1-binding activity is a property of the quaternary but not the monomer. Provided that a suitable cellular environment and chaperones exist, folding and assembly of CT-B in plant tissues follow analogous pathways to those observed in the bacterial system. The oxidative environment of the ER, and the abundance of protein folding chaperones therein that facilitate formation of disulfide bonds, make it particularly amenable to pentamer formation. Thus, the majority of the T3 optimization strategies consist in targeting CT-B to the ER using signal peptides and retention tags such as KDEL. Assessment of pentamer formation is also commonly performed via GM1-ELISA and non-reducing SDS-PAGE, which can differentiate between fully assembled pentamers versus incompletely assembled or misfolded subunits [52]

3. **Assessment of GM1 Receptor-Binding Affinity of Plant-Derived CT-B**
GM1-binding assays are the most accurate way to check the bioactivity of the protein, as they reflect the binding affinity and hence the correct folding and assembly of the protein. When expressed under optimal conditions, plant-derived CT-B has been strong and consistent in its GM1 binding affinity comparable to that of bacterial preparations. Differences in binding efficiency can be attributed to certain changes after translation specific to the plant, such as glycosylation, which may change the protein surface properties. Nevertheless, the CT-B structure does not contain any necessary glycosylation sites and it is quite stable, so it is hard to be disrupted by modifications from plants. Even if glycosylation takes place, it is in most cases very little and thus does not noticeably interfere with receptor binding, though this aspect must be considered for each plant species and expression system independently [53-54]

4. **Functional Bioactivity Evaluation Through Mucosal Interaction Studies**
Antigen stability in plants is also a very important factor that determines the overall feasibility of vaccine production. The CT-B protein must be able to resist degradation inside cells, be stable in different environmental conditions during the growing period, and also be resistant to further processing steps like harvesting, drying, or storage. The stability of a protein is largely dependent on its subcellular localisation. Moreover, if the protein is imported to the chloroplasts, it can become very stable because of the high protein storage capacity and least exposure to the degradative processes; however, apart from that, the formation of the correct disulfide bond still needs to be verified carefully in this area [55].

5. **Influence of Storage, Environmental Conditions, and Processing Methods on CT-B Integrity**
Determinations of stability after harvesting most commonly include evaluations of antigen retention in plant tissues under different storage conditions. Getting rid of moisture (lyophilisation) in the case of edible plant tissues has been recognised as one

of the most effective means of stabilising CT-B over long periods and under non-refrigerated conditions. Lyophilised tissues protect protein structure by minimising moisture-dependent degradation and provide a straightforward way of converting the protein into powdered or capsule-containing oral formulations. Stability may be further confirmed by accelerated ageing experiments, where degradation occurring at high temperatures is measured to simulate the conditions of the distribution of vaccines in the tropics. Such tests are important in determining the shelf life of plant-derived CT-B and the potential for its use in vaccine production in areas where the cold-chain is unavailable [56].

IMMUNOGENICITY OF PLANT-DERIVED CT-B IN PRECLINICAL STUDIES

Immunogenic potential of a plant-derived cholera toxin B subunit (CT-B) has been the major focus of extensive research in preclinical systems, with the establishment of its suitability as a mucosal antigen to be delivered orally. CT-B is in essence a great candidate for oral immunization because its pentameric structure interacts very tightly with GM1 ganglioside receptors which are situated on intestinal epithelial and antigen-presenting cells. The interaction thus allows uptake, transcytosis, and presentation via mucosal immune pathways to be done effectively [57]. Data from plant-expressed CT-B studies have shown that the antigen when is produced and folded properly, especially retaining pentamer formation and GM1-binding activity, is capable of mucosal immune system induction in a very low adjuvant scenario or even an absence one. Oral immunogenicity of CT-B in edible plants was primarily tested in rodent models such as mice and rats. The plants included potato, tomato, rice, lettuce, and maize [58]. Some of these studies are also experiments with feeding of freeze-dried plant tissue, fresh leaf material, or purified plant-derived CT-B. One of the most critical points in numerous experiments is that the protein synthesis of CT-B in plant cell walls makes the protein more resistant to gastric acid and proteolytic degradation. This natural bio-encapsulation makes available more antigen for the small intestine, where it can interact with mucosal surfaces. Very low doses (10–50 µg of CT-B equivalents) have been able to achieve measurable immune responses after repeated oral exposure, thus the concept of functional antigen delivery through plant tissues has been successfully demonstrated in a number of preclinical studies [59].

The signature feature of CT-B's function is probably its capacity to evoke IgA responses at mucosal level. It results from the GM1- binding property. In preclinical feeding trials, recombinant CT-B was found to be very effective in eliciting antigen-specific secretory IgA in different biological fluids such as intestinal lavage, fecal extracts, and saliva [60]. Those reflect activation of mucosal-associated lymphoid tissue (MALT) which is mainly Peyer’s patches where after CT-B internalization plasma cells secreting IgA are differentiated. In several experimental settings, mucosal responses were on the same level as those brought about by purified bacterial CT-B if the plant-derived antigen was retained in the proper folds and was correctly assembled. The degree of mucosal immunity was also found to depend on how much

CT-B was accumulated in the plant tissue, the mode of delivery (fresh or freeze-dried) and the co-administration schedules.

Apart from mucosal IgA, plant-derived CT-B has also been linked to systemic IgG responses particularly IgG1 and IgG2a subclasses. This dual profile of the response signals that oral CT-B can activate both Th1 and Th2 pathways and this depends on dosing frequency and physiologic condition of the antigen Systemic responses are mostly necessitated by several dosages but promote rapidly if one is carrying out high-level expression of CT-B, endoplasmic reticulum targeting, or rice endosperm storage stabilization [61]. One paper stated that CT-B oral administration induced systemic neutralizing antibodies, which in turn, could inhibit cholera toxin-mediated fluid secretion in mouse intestinal loop assays, showing how the system works. On the molecular level, the plant CT-B is similar to the native CT-B as it obtains the same pathways. They include GM1-mediated uptake, retrograde transport, as well as interaction with dendritic cells and B cells within GALT. The pentameric structure is of importance for receptor binding and the ability to evoke immune responses; monomeric forms do not have this capability [62]. In most of the preclinical trials CT-B served the role of both an antigen and a mucosal adjuvant thereby resulting in a greater immune response to the co-expressed antigens such as heat-labile toxin B (LT-B) or bacterial flagellin. This feature of CT-B renders it a desirable ingredient in multivalent edible vaccine platforms.

ADVANTAGES, CONSTRAINTS, AND BIOSAFETY CONSIDERATIONS

The invention of plant-based cholera toxin B subunit (CT-B) vaccines represents a revolutionary way to immunize orally with a combination of low-cost production, high scalability, and the intrinsic safety of non-replicating antigen systems. Edible plant platforms deliver a novel way to elicit mucosal immunity via the oral route, thus overcoming the obstacles of global health posed by intestinal diseases like cholera [63]. But, besides these advantages, there are several limitations, safety concerns, and complicated regulations that need to be addressed before the realization and safety of this technology can be confirmed. One of the notable benefits of plant systems for CT-B production is the drastic reduction in the cost of the whole process [64]. In contrast to bioreactors based on fermentation, plant systems do not need a lot of money to set up the facility as they are essentially based on farming. That makes them very suitable for the places with poor resources, where the manufacture of traditional vaccines is too expensive. Moreover, plants are not capable of facilitating the replication of human pathogens, thus, the fears of contamination by viruses, endotoxins, or prions that are usually the result of microbial or mammalian systems are completely ruled out [65]. Scalability is also a major point. The amount of plant material can be produced locally or seasonally and extended out district-wise once a stable transgenic line is obtained. Besides, for instance, the transformation of chloroplasts can achieve extraordinarily high antigen levels, sometimes reaching 70% of the total soluble protein in the initial experiment model. Such a high level of expression significantly broadens the application of oral vaccines derived from plants and shortens the downstream processing tasks [66].

Firstly, the expression of CT-B is highly variable in different plant tissues, developmental stages, and environmental conditions. Technically it is very difficult to accomplish high levels of CT-B all the time, especially in edible species which do not tolerate strong promoters or high metabolic burdens [67]. Besides, edible plant tissues usually have low protein content, so large amounts may be needed for immunisation. Moreover, there is a limitation associated with post-translational modifications. Even if CT-B does not need glycosylation for correct folding and assembling, the differences between plant and bacterial systems may affect antigen stability or immune recognition [68]. On the other hand, anti-GM1 binding and mucosal immunogenicity require that the correct pentamer formation is ensured, however, oxidative stress, high-temperature storage, and prolonged processing may lead to improper folding. Besides, dosage control is another limitation. Edible plant vaccines may have different antigen contents per unit weight with no possibility of measuring purification, thus making standardisation required for clinical use difficult [69]. Producing freeze-dried capsules or tablets from tissues can alleviate this variability; however, it complicates the formulation. Public attitude to genetic modification constitutes a real constraint. Although genetically modified plants have been scientifically proven to be safe, consumers concerns about GMOs foods could delay its diffusion especially in countries with a strong anti-GMO stance [70].

FUTURE PRESPECTIVES IN EDIBLE PLANT BASED CHOLERA IMMUNIZATIONS

The innovation of edible plant-based cholera vaccines is a major shift in the worldwide immunisation system, especially in areas with limited resources, where maintaining the cold chain, multiple clinical visits, and high production costs make vaccine access difficult. The use of an edible plant expressing the B subunit of the cholera toxin (CT-B) as an antigen is a very attractive solution because it combines very cheap production with oral administration and mucosal immune targeting [71]. While the development of plant-based CT-B has gone a long way, there are still a bunch of far-reaching ideas, translational opportunities, and tactical moves that can help bring these platforms to clinical and public health levels. A significant breakthrough that the research could lead to is the use of advanced genetic engineering tools in the work, more particularly, precision editing through CRISPR/Cas. CRISPR permits highly targeted changes in the promoter strength, codon usage, and post-translational modification pathways, all of which can have a very large impact on how much CT-B is expressed and how stable it is [72]. On top of that synthetic biology paradigms, modular expression cassettes can be created to regulation transcriptional strength, mRNA stability, protein folding, and even control the tissue-specific expression at a very fine level. The concept of plant-based biofoundries is becoming more and more highly automated with a very large number of different approaches being tested simultaneously thus very rapid optimisation cycles which were previously impossible can be performed nowadays [73]. Furthermore, the use of plant viral vectors capable of a transient but very high expression level may pave the way for the swift production of CT-B antigen stockpiles in the case of a sudden outbreak.

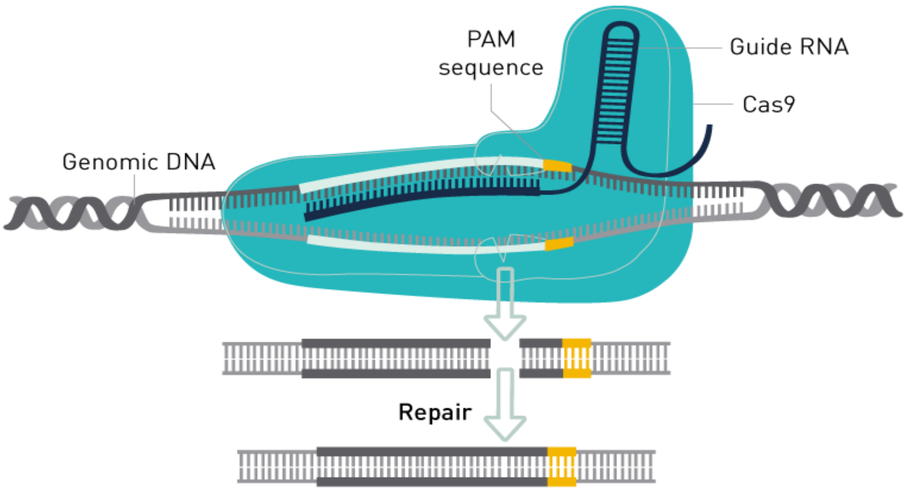

Fig 4. Genome editing (CRISPR/Cas9)

Equally, the evolution of new-generation edible plant bases is of great significance. The initial studies mainly concentrated on species like potato, tomato, rice, and lettuce, but the next research will probably extend to the crops that have better shelf-stability, are more adaptable to the climate, and have high biomass [74]. Among the proposed candidates, bananas, maize, wheat, and microgreens stand out due to their very simple consumption, worldwide distribution, and the ability to keep the antigens intact during storage. Hydroponic plant systems based on bioreactors represent the next step, where growth conditions can be very precisely controlled and the risk of environmental contamination can be minimal. These closed setups might be very useful especially for fulfilling the requirements of the regulatory and biosafety standards and at the same time allowing continuous production [75].

Besides production, delivery optimisation is still a very important task that can be solved with future innovations. By itself, CT-B is able to strongly bind the GM1 ganglioside receptor and therefore is a good candidate for mucosal vaccine delivery but its immunogenicity can be even more improved by formulation strategies. Oral uptake and antigen presentation can be enhanced to a great extent if the methods such as microencapsulation in the plant cell wall matrices, using mucosal adjuvants, or preparing fusion constructs with pathogen-associated molecular patterns are applied [76]. The progress in drying, milling, and encapsulation techniques can pave the way for the development of powder-form edible vaccines that are stable at room temperatures, can be easily transported, and are fit for incorporation into fortified food products. These types of innovations are very much in line with global health goals which aim at providing scalable and field-ready vaccine formats. From a translational perspective, the clinical evaluation pipeline of CT-B vaccines made from plants will probably be widened as regulatory agencies provide more clear-cut guidelines for pharmaceuticals from plants [77]. The move of candidates to clinical trials will largely depend on the development of the standardised assays for antigen quantification, folding verification, pentamer formation, and

GM1-binding alignment. And harmonized biosafety assessments (taking into account pollen flow, genetic containment and environmental release) will lead to less restricted outdoor production or controlled environment production of plant-derived pharmaceuticals. International partnerships may accelerate these developments, particularly in such areas as South Asia and sub-Saharan Africa, where cholera is an endemic and epidemic disease [78].

One more area of growth could be the usage of edible CT-B vaccines integrated with public health and vaccination programs. In fact, plant-derived antigens could be used as booster doses after a primary immunisation with injectable cholera vaccines, thus ensuring the continuation of mucosal immunity. Distribution through schools, fortified food programs, and community nutrition initiatives may turn out to be effective ways of reaching the most vulnerable populations, such as children and rural communities. Decentralized manufacturing using rapid production technologies could therefore serve as a vehicle for delivering vaccines quickly in locales where traditional vaccine supplies are strained by outbreaks [79]. Furthermore, genome-encoded vaccines in perennial edible crops, plant-based nanoparticle scaffolds and multivalent edible vaccine constructs comprising CT-B linked with antigens for enterotoxigenic E. coli (ETEC) and/or rotavirus could emerge as the future of oral immunisation regimens. Indeed, the convergence of plant biotechnology, mucosal immunology, and global health systems design may herald a future where vaccines not only become vastly more attainable but also quietly slip into the fabric of global nutritional and public health systems [80].

CONCLUSION

The generation of edible plant-based systems for the production and delivery of Cholera Toxin B subunit (CT-B) is a major, hopeful shift in the oral immunisation sector. One of the major themes that have emerged from the literature over these years is that plant tissues expressing CT-B can keep the vital structural and functional properties such as pentamer formation, GM1-ganglioside receptor binding, and the ability to induce mucosal and systemic immunity. These are proofs of concept food crops can be used as vaccine biofactories.

While cholera is still a major problem for the poor in the least developed countries, the necessity of vaccines that are cheap, can be easily scaled up and administered orally is still pressing. Why not decentralise vaccine production through plant-derived CT-B? This way we can eliminate many of the logistical and economic barriers that are the hallmarks of cold chain- and fermentation–dependent systems. The maximisation of plant-based CT-B expression by means of transgene engineering, codon optimisation, promoter selection, subcellular targeting, and protein stabilisation strategies has very much pumped up antigen yield and hence ensured that the work is of practical relevance on a large scale. A lot of progress has been made in chloroplast transformation, ER-retention methods, fusion peptide engineering, and plant viral vectors to raise CT-B to the level of functional immunogenic evaluations. Meanwhile, numerous preclinical studies point to oral delivery of plant tissue-encapsulated CT-B whereby the natural cell walls protect the antigen from gastrointestinal degradation and at the same time allow for controlled release at mucosal sites. The inherent advantages serve to confirm that edible plants are the best delivery vehicles in that they not only facilitate the activation of gut-associated lymphoid tissue (GALT) but also improve mucosal immune responses-which constitute the mainstay of cholera protection.

Notwithstanding these successes, there are still a few limitations and biosafety concerns. Among the factors that contribute to variance in the expression of antigens from plant to plant are transgene escapes, and the presence of various regulatory frameworks for genetic modification in different parts of the world. There is a high possibility that dose standardisation will remain difficult because of biological variability which is a characteristic feature of agricultural systems. The acceptance of genetically modified edible vaccines by the public is also dependent on good communication, transparency, and risk assessment. If these problems are solved, it will be easier to get both regulatory and societal greenlighting. The next moves in gene editing, plant synthetic biology, transient expression systems, and molecular farming technologies might still be able to solve most of the problems that are currently stalling the field. Plant-produced CT-B vaccines of the future might be very close to commercial maturity if there is a successful interplay between CRISPR-based genomic control, edible plant plastid engineering, nano-biotechnology enhancements, and thermostable antigen platforms. Besides that, coupling CT-B with other antigens, microbial adjuvants, or multivalent oral vaccine designs could extend applications beyond cholera to integrated diarrheal disease prevention. To put it briefly, the work done on plant expression of CT-B is the breakthrough that oral vaccines accessible to everybody and safe for them have long needed. The refinement of techniques, clearance of regulations and validation at a large scale still lie ahead but the firm scientific basis and the technological progress give the impression that plant-derived CT-B may be an important element of future global immunisation strategies, especially in the areas where the infrastructure for conventional vaccines is hardly existent. Taken together, the evidence is in line with the idea that edible plant-based cholera immunisation is a powerful tool for global public health resilience.

REFERENCES:

1. Pollitzer R. Cholera. Geneva: World Health Organization; 1959. https://pubmed.ncbi.nlm.nih.gov/13160764/
2. Clemens JD, Nair GB, Ahmed T, Qadri F, Holmgren J. Cholera. Lancet. 2017;390(10101):1539–49. doi: http://doi.org/10.1016/S0140-6736(17)30559-7.
3. Harris JB, LaRocque RC, Qadri F, Ryan ET, Calderwood SB. Cholera. Lancet. 2012;379(9835):2466–76. doi: http://doi.org/10.1016/S0140-6736(12)60436-X.
4. Holmgren J, svennerholm AM, Lonnroth, I. *et al.* Development of improved cholera vaccine based on subunit toxoid. *Nature* **269,** 602–604 (1977). https://doi.org/10.1038/269602a0
5. Sanchez J, Holmgren J. Cholera toxin structure, genetics, and function. Microbiol Rev. 1989;53(3):403–15. https://pubmed.ncbi.nlm.nih.gov/18278577/
6. Tacket CO, Mason HS, Losonsky G, Clements JD, Levin MJ, Arntzen CJ. Immunogenicity of recombinant Vibrio cholerae B subunit produced in transgenic potatoes. Nat Med. 1998;4(5):607–9. doi: http://doi.org/10.1038/nm0598-607.

7. Wernick NLB, Chinnapen DJF, Cho JA, Lencer WI. Cholera toxin: an intracellular journey into the cytosol by way of the endoplasmic reticulum. Toxins. 2010;2(3):310-325. http://doi.org/10.3390/toxins2030310.
8. Kurokawa S, Fang J, Shaheen S, Hikichi Y, Inagaki S, Takaku H. Oral vaccination with transgenic rice expressing cholera toxin B subunit induces protective immune responses. Vaccine. 2023;41(10):1624–31. doi: http://doi.org/10.1016/j.vaccine.2023.01.023.
9. Rosales-Mendoza S, Angulo C, Meza B. Food-grade vaccines: Current status and prospects. Infect Genet Evol. 2016;40:124–38. doi: https://pubmed.ncbi.nlm.nih.gov/26708345/ .
10. Chan HT, Xiao Y, Weldon WC, Oberste SM, Chumakov K, Daniell H. Cold chain–free orally delivered plant-based vaccine protects against poliovirus. Plant Biotechnol J. 2016;14(10):2190–200. doi: https://pmc.ncbi.nlm.nih.gov/articles/PMC6576100/ .
11. Rybicki EP. Plant-based vaccines against viruses. Virol J. 2014;11:205. doi: http://doi.org/10.1186/s12985-014-0205-0
12. Mason HS, Lam DM, Arntzen CJ. Expression of hepatitis B surface antigen in transgenic plants. Proc Natl Acad Sci U S A. 1992;89(20):11745–9. doi: https://pubmed.ncbi.nlm.nih.gov/1465391/.
13. Streatfield SJ. Approaches to achieve high-level heterologous protein production in plants. Plant Biotechnol J. 2007;5(1):2–15. doi: http://doi.org/10.1111/j.1467-7652.2006.00216.x .
14. Yusibov V, Kushnir N, Streatfield SJ. Antibody production in plants and green algae. Annu Rev Plant Biol. 2016;67:669–701. doi: https://pubmed.ncbi.nlm.nih.gov/26905655/.
15. Takeyama N, Kiyono H, Yuki Y. Plant-based vaccines for gastrointestinal infectious diseases: Salmonella, enterohemorrhagic E. coli, norovirus, and rotavirus. Front Microbiol. 2015;5:1–9. doi: https://pubmed.ncbi.nlm.nih.gov/26668752/.
16. Mett V, Musiychuk K, Bi H, Farrance CE, Horsey A, Ugulava N, et al. A plant-produced influenza subunit vaccine protects ferrets against virus challenge. Influenza Other Respir Viruses. 2008;2(1):33–40. doi: https://pmc.ncbi.nlm.nih.gov/articles/PMC4634330/.
17. Kapusta J, Modelska A, Figlerowicz M, Pniewski T, Letellier M, Lisowa O, et al. A plant-derived edible vaccine against hepatitis B virus. FASEB J. 1999;13(13):1796–9. doi: http://doi.org/10.1096/fasebj.13.13.1796.
18. Arntzen CJ. Plant-made pharmaceuticals: From “Edible Vaccines” to Ebola therapeutics. Plant Biotechnol J. 2015;13(8):1013–6. doi: https://pmc.ncbi.nlm.nih.gov/articles/PMC11962629/
19. Pniewski T. Plant-based vaccines against hepatitis B. Wiley Interdiscip Rev Nanomed Nanobiotechnol. 2013;5(3):332–41. doi: https://pubmed.ncbi.nlm.nih.gov/20673016/.
20. Chan HT, Daniell H. Plant-made oral vaccines against human infectious diseases—Are we there yet? Plant Biotechnol J. 2021;19(9):1755–76. doi: https://pubmed.ncbi.nlm.nih.gov/26387509/ .

21. Rigano MM, Walmsley AM. Expression systems and developments in plant-made vaccines. Immunol Cell Biol. 2005;83(3):271–7. doi: https://pubmed.ncbi.nlm.nih.gov/15877605/ .
22. Spangler BD. Structure and function of cholera toxin and the related Escherichia coli heat-labile enterotoxin. https://pubmed.ncbi.nlm.nih.gov/1480112/
23. Sixma TK, Pronk SE, Kalk KH, Wartna ES, van Zanten BA, Witholt B, Hol WG. Crystal structure of a cholera toxin-related heat-labile enterotoxin from E. coli. Nature. 1991;351(6325):371–7. doi: https://pubmed.ncbi.nlm.nih.gov/2034287/
24. Zhang RG, Scott DL, Westbrook ML, Nance S, Spangler BD, Shipley GG, Westbrook EM. The three-dimensional crystal structure of cholera toxin. J Mol Biol. 1995;251(4):563–73. doi: https://pubmed.ncbi.nlm.nih.gov/7658473/.
25. Lencer WI, Saslowsky D. Raft trafficking of the cholera toxin B subunit: role of ganglioside GM1. Adv Exp Med Biol. 2005;560:37–52. doi: https://pubmed.ncbi.nlm.nih.gov/1651376/.
26. Merritt EA, Sixma TK, Kalk KH, van Zanten BA, Hol WG. Galactose-binding site in Escherichia coli heat-labile enterotoxin (LT) B-pentamer. Nat Struct Biol. 1994;1(10):727–34. doi: https://pubmed.ncbi.nlm.nih.gov/7997185/.
27. Kuziemko GM, Stroh M, Stevens RC. Cholera toxin binding affinity and specificity for gangliosides determined by surface plasmon resonance. Biochemistry. 1996;35(20):6375–84. doi: https://pubmed.ncbi.nlm.nih.gov/8639583/.
28. Sánchez J, Holmgren J. Similarities in structure and function between cholera toxin and heat-labile enterotoxins. Microbiol Immunol. 2008;52(8):311–20. doi: https://pmc.ncbi.nlm.nih.gov/articles/PMC264483/.
29. Millar DG, Hirst TR. Cholera toxin B-subunit: an effective mucosal adjuvant for oral and nasal immunization. Immunology. 1992;75(3):527–32. https://pubmed.ncbi.nlm.nih.gov/8256498/
30. Lycke N. Cholera toxin promotes thymus-dependent and independent mucosal immune responses. Adv Exp Med Biol. 1995;371B:1403–8. doi: https://pmc.ncbi.nlm.nih.gov/articles/PMC1453161/.
31. Mbongue JC, Vanterpool E, Langridge WHR. Exploring the potential of plant-based CTB-INS oral vaccines in treating type 1 diabetes. *Immuno.* 2023;3(2):217-227. http://doi.org/10.3390/immuno3020014.
32. Kwon KC, Chan HT, León IR, Williams-Carrier R, Barkan A, Daniell H. Codon optimization to enhance expression yields insights into chloroplast translation. Plant Physiol. 2016;172(1):62-77. http://doi.org/10.1104/pp.16.00981.
33. Daniell H, Lee SB, Panchal T, Wiebe PO. Expression of the native cholera toxin B subunit gene and assembly as functional oligomers in transgenic tobacco chloroplasts. J Mol Biol. 2001;311(5):1001-1009. http://doi.org/10.1006/jmbi.2001.4921.
34. Jertborn M, Svennerholm AM, Holmgren J. Saliva, breast milk, and serum antibody responses as indirect measures of intestinal immunity after oral cholera vaccination or natural disease. J Clin Microbiol. 1986;24(2):203-209. http://doi.org/10.1128/jcm.24.2.203.
35. Hamorsky KT, Kouokam JC, Bennett LJ, Baldauf KJ, Kajiura H, Fujiyama K, Matoba N. 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(3):e2046. http://doi.org/10.1371/journal.pntd.0002046.

36. Su H, Rong G, Li L, Cheng Y. Subcellular targeting strategies for protein and peptide delivery. Adv Drug Deliv Rev. 2024;212:115387. http://doi.org/10.1016/j.addr.2024.115387.
37. Chebolu S, Daniell H. Chloroplast-derived vaccine antigens and biopharmaceuticals: expression, folding, assembly and functionality. Curr Top Microbiol Immunol. 2009;332:33-54. http://doi.org/10.1007/978-3-540-70868-1_3.
38. Mishra S, Yadav DK, Tuli R. Ubiquitin fusion enhances cholera toxin B subunit expression in transgenic plants and the plant-expressed protein binds GM1 receptors more efficiently. J Biotechnol. 2006 Dec 15;127(1):95-108. http://doi.org/10.1016/j.jbiotec.2006.06.002.
39. Baldauf KJ, Royal JM, Hamorsky KT, Matoba N. Cholera toxin B: one subunit with many pharmaceutical applications. Toxins (Basel). 2015 Mar 20;7(3):974-96. http://doi.org/10.3390/toxins7030974.
40. Dolan JP, Machin DC, Dedola S, Field RA, Webb ME, Turnbull WB. Synthesis of cholera toxin B subunit glycoconjugates using site-specific orthogonal oxime and sortase ligation reactions. Front Chem. 2022 Sep 14;10:958272. http://doi.org/10.3389/fchem.2022.958272.
41. Giddings G, Allison G, Brooks D, Carter A. Transgenic plants as factories for biopharmaceuticals. Nat Biotechnol. 2000;18:1151-1155. http://doi.org/10.1038/81132.
42. Arakawa T, Chong DK, Langridge WH. Efficacy of a food plant-based oral cholera toxin B subunit vaccine. Nat Biotechnol. 1998;16(3):292-297. http://doi.org/10.1038/nbt0398-292.
43. Jani D, Meena LS, Rizwan-ul-Haq QM, Singh Y, Sharma AK, Tyagi AK. Expression of cholera toxin B subunit in transgenic tomato plants. Transgenic Res. 2002 Oct;11(5):447-54. doi: http://doi.org/10.1023/a:1020336332392
44. Oszvald M, Kang TJ, Tomoskozi S, Jenes B, Kim TG, Cha YS, Tamas L, Yang MS. Expression of cholera toxin B subunit in transgenic rice endosperm. Mol Biotechnol. 2008 Nov;40(3):261-8. doi: http://doi.org/10.1007/s12033-008-9083-2
45. Loc N, Thinh L, Yang M, et al. Highly expressed cholera toxin B subunit in the fruit of a transgenic tomato (Lycopersicon esculentum L.). Biotechnol Bioprocess Eng. 2011;16(3):576-580. doi: http://doi.org/10.1007/s12257-010-0195-8
46. Tacket CO, Mason HS, Losonsky G, Clements JD, Levine MM, Arntzen CJ. Immunogenicity in humans of a recombinant bacterial antigen delivered in a transgenic potato. Nat Med. 1998;4(5):607-609. doi: http://doi.org/10.1038/nm0598-607
47. Hamorsky KT, Kouokam JC, Bennett LJ, Baldauf KJ, Kajiura H, Fujiyama K, Matoba N. 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(3):e2046. doi: http://doi.org/10.1371/journal.pntd.0002046
48. Soh HS, Chung HY, Lee HH, et al. Expression and functional validation of heat-labile enterotoxin B (LTB) and cholera toxin B (CTB) subunits in transgenic rice (Oryza sativa). SpringerPlus. 2015;4:148. doi: http://doi.org/10.1186/s40064-015-0847-4

49. 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(3):527-537. doi: http://doi.org/10.1007/s00299-011-1146-3
50. Jiang XL, He ZM, Peng ZQ, Qi Y, Chen Q, Yu SY. Cholera toxin B protein in transgenic tomato fruit induces systemic immune response in mice. Transgenic Res. 2007;16(2):169-175. doi: http://doi.org/10.1007/s11248-006-9023-5
51. Delmer D, Dixon RA, Keegstra K, Mohnen D. The plant cell wall—dynamic, strong, and adaptable—is a natural shapeshifter. Plant Cell. 2024;36(5):1257-1311. doi: http://doi.org/10.1093/plcell/koad325
52. Kwon KC, Nityanandam R, New JS, Daniell H. Oral delivery of bioencapsulated exendin-4 expressed in chloroplasts lowers blood glucose in mice and stimulates insulin secretion in beta-TC6 cells. Plant Biotechnol J. 2013;11(1):77-86. doi: http://doi.org/10.1111/pbi.12008
53. Jobling MG, Holmes RK. Mutational analysis of ganglioside GM1-binding ability, pentamer formation, and epitopes of cholera toxin B subunits and CTB/LTB chimeras. Infect Immun. 2002;70(3):1260-71. doi: http://doi.org/10.1128/IAI.70.3.1260-1271.2002
54. Worstell NC, Krishnan P, Weatherston JD, Wu HJ. Binding cooperativity matters: a GM1-like ganglioside–cholera toxin B subunit binding study using a nanocube-based lipid bilayer array. PLoS One. 2016;11(4):e0153265. doi: http://doi.org/10.1371/journal.pone.0153265
55. Vaou N, Stavropoulou E, Voidarou CC, Tsakris Z, Rozos G, Tsigalou C, Bezirtzoglou E. Interactions between medical plant-derived bioactive compounds: focus on antimicrobial combination effects. Antibiotics (Basel). 2022;11(8):1014. doi: http://doi.org/10.3390/antibiotics11081014
56. DeBenedictis JN, de Kok TM, van Breda SG. Impact of processing method and storage time on phytochemical concentrations in an antioxidant-rich food mixture. Antioxidants (Basel). 2023;12(6):1252. doi: http://doi.org/10.3390/antiox12061252
57. Thanavala Y, Yang YF, Lyons P, Mason HS, Arntzen C. Immunogenicity of transgenic plant-derived hepatitis B surface antigen. Proc Natl Acad Sci U S A. 1995;92(8):3358-3361. doi: http://doi.org/10.1073/pnas.92.8.3358
58. Phoolcharoen W, Shanmugaraj B, Khorattanakulchai N, Sunyakumthorn P, Pichyangkul S, Taepavarapruk P, Praserthsee W, Malaivijitnond S, Manopwisedjaroen S, Thitithanyanont A, Srisutthisamphan K, Jongkaewwattana A, Tomai M, Fox CB, Taychakhoonavudh S. Preclinical evaluation of immunogenicity, efficacy and safety of a recombinant plant-based SARS-CoV-2 RBD vaccine formulated with 3M-052-Alum adjuvant. Vaccine. 2023;41(17):2781-2792. doi: http://doi.org/10.1016/j.vaccine.2023.
59. Shanmugaraj B, Khorattanakulchai N, Panapitakkul C, Malla A, Im-Erbsin R, Inthawong M, Sunyakumthorn P, Hunsawong T, Klungthong C, Reed MC, Kemthong T, Suttisan N, Malaivijitnond S, Srimangkornkaew P, Klinkhamhom A, Manopwisedjaroen S, Thitithanyanont A, Taychakhoonavudh S, Phoolcharoen W. Preclinical evaluation of a plant-derived SARS-CoV-2 subunit vaccine: protective efficacy, immunogenicity, safety, and toxicity. Vaccine. 2022;40(32):444. http://doi.org/10.1016/j.vaccine.2022.05.087

60. Doroud D, Daneshi M, Kazemi-Lomedash F, Eftekhari Z. Comprehensive review of preclinical evaluation strategies for COVID-19 vaccine candidates: assessing immunogenicity, toxicology, and safety profiles. Iran J Microbiol. 2025;17(1):1-18. doi: http://doi.org/10.18502/ijm.v17i1.
61. Kumar A, Sharma A, Tirpude NV, Padwad Y, Hallan V, Kumar S. Plant-derived immuno-adjuvants in vaccines formulation: a promising avenue for improving vaccines efficacy against SARS-CoV-2 virus. Pharmacol Rep. 2022;74(6):1238-1254. doi: http://doi.org/10.1007/s43440-022-00418-
62. Green MD, Al-Humadi NH. Preclinical toxicology of vaccines. In: A Comprehensive Guide to Toxicology in Nonclinical Drug Development. 2017:709–735. doi: http://doi.org/10.1016/B978-0
63. Streatfield SJ, Jilka JM, Hood EE, Turner DD, Bailey MR, Mayor JM, Woodard SL, Beifuss KK, Horn ME, Delaney DE, Tizard IR, Howard JA. Plant-based vaccines: unique advantages. Vaccine. 2001;19(17-19):2742-2748. doi: http://doi.org/10.1016/S0264-410X(00)00512-0
64. Czerkinsky C, Svennerholm AM, Holmgren J. Induction and assessment of immunity at enteromucosal surfaces in humans: implications for vaccine development. Clin Infect Dis. 1993;16(Suppl 2):S106-116. doi: http://doi.org/10.1093/clinids/16.supplement_2.s106
65. Ishida Y, Saito H, Ohta S, Hiei Y, Komari T, Kumashiro T. High efficiency transformation of maize (Zea mays L.) mediated by Agrobacterium tumefaciens. Nat Biotechnol. 1996;14(6):745-750. https://pubmed.ncbi.nlm.nih.gov/9630983/
66. Takeyama N, Kiyono H, Yuki Y. Plant-based vaccines for animals and humans: recent advances in technology and clinical trials. Ther Adv Vaccines. 2015;3(5-6):139-154. doi: http://doi.org/10.1177/205101361561
67. Venkataraman S, Hefferon K, Makhzoum A, Abouhaidar M. Combating human viral diseases: will plant-based vaccines be the answer? Vaccines (Basel). 2021;9(7):761. doi: http://doi.org/10.3390/vaccines9070761
68. Chan HT, Daniell H. Plant-made oral vaccines against human infectious diseases—are we there yet? Plant Biotechnol J. 2015;13(8):1056-70. doi: http://doi.org/10.1111/pbi.12471
69. Saxena J, Rawat S. Edible vaccines. Advances in Biotechnology. 2013:207–26. doi: http://doi.org/10.1007/978-81-322-1554-7_12
70. Hamorsky KT, Kouokam JC, Bennett LJ, Baldauf KJ, Kajiura H, Fujiyama K, Matoba N. 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(3):e2046. doi: http://doi.org/10.1371/journal.pntd.000204
71. Siamalube B, Ehinmitan E, Kachenga L, Runo S, Ngotho M, Onguso J. Plant-based edible vaccines: Can cholera be the case study in Africa? J Genet Eng Biotechnol. 2025 Sep;23(3):100527. doi: http://doi.org/10.1016/j.jgeb.2025.100527
72. Concha C, Cañas R, Macuer J, Torres MJ, Herrada AA, Jamett F, Ibáñez C. Disease Prevention: An Opportunity to Expand Edible Plant-Based Vaccines? Vaccines (Basel). 2017 May 30;5(2):14. doi: http://doi.org/10.3390/vaccines5020014

73. Sahai A, Shahzad A, Shahid M. Plant Edible Vaccines: A Revolution in Vaccination. Recent Trends in Biotechnology and Therapeutic Applications of Medicinal Plants. 2013 Apr 15:225–52. doi: http://doi.org/10.1007/978-94-007-6603-7_10
74. Qureshi MB, Arif S, Rathore SS. Edible plant vaccines: a step towards revolution in the field of immunology. Int J Agric Biol. 2023;29(5):361-369. doi: http://doi.org/10.17957/IJAB
75. https://www.diagenode.com/en/categories/crispr-cas9-genome-editing
76. Khalid F, Tahir R, Ellahi M, Amir N, Rizvi SFA, Hasnain A. Emerging trends of edible vaccine therapy for combating human diseases especially COVID-19: Pros, cons, and future challenges. Phytother Res. 2022 Jul;36(7):2746-2766. doi: http://doi.org/10.1002/ptr.7475
77. Kurup VM, Thomas J. Edible Vaccines: Promises and Challenges. Mol Biotechnol. 2020 Feb;62(2):79-90. doi: http://doi.org/10.1007/s12033-019-00222-1.
78. Sahoo A, Mandal AK, Dwivedi K, Kumar V. A cross talk between the immunization and edible vaccine: Current challenges and future prospects. Life Sci. 2020 Nov 15;261:118343. doi: http://doi.org/10.1016/j.lfs.2020.118343.
79. Gunasekaran B, Gothandam KM. A review on edible vaccines and their prospects. Braz J Med Biol Res. 2020 Jan 24;53(2):e8749. doi: http://doi.org/10.1590/1414-431X20198749.
80. Munshi A, Sharma V. Omics and Edible Vaccines. In: Omics Technologies and Bio-Engineering. 2018:129–41. doi: http://doi.org/10.1016/B978-0-12-815870-8.00008-5.