Trends in Biotechnology

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Review

Green Therapeutic Biocapsules: Using Plant Cells to Orally Deliver Biopharmaceuticals

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The use of innovative platforms to produce biopharmaceuticals cheaply and deliver them through noninvasive routes could expand their social benefits. Coverage should increase as a consequence of lower cost and higher patient compliance due to painless administration. For more than two decades of research, oral therapies that rely on genetically engineered plants for the production of biopharmaceuticals have been explored to treat or prevent high-impact diseases. Recent reports on the successful oral delivery of plant-made biopharmaceuticals raise new hopes for the field. Several candidates have shown protection in animal models, and efforts to establish their production on an industrial scale are ongoing. These advances and perspectives for the field are analyzed.

Highlights

Plant-made biopharmaceuticals have been researched for more than two decades, but such medicines are not yet commercially available.

Recent advances have improved the oral delivery potential of plant-made biopharmaceuticals.

Oral delivery of biopharmaceuticals using the B subunit of cholera toxin as a transmucosal carrier has brought new hope to this field.

The development of oral plant-based vaccines still requires addressing some regulatory and technical aspects.

Plant Cells and Oral Biopharmaceutical Delivery

The advent of recombinant DNA technologies led to the development of platforms for the production of recombinant **biopharmaceuticals** (see Glossary), which are complex molecules produced in a genetically modified organism used as an expression host. Biopharmaceuticals have prevented millions of deaths as they delay or reverse pathologies or provide protection against infections through highly specific interactions with their targets. For instance, the first recombinant vaccine used in humans has saved millions of deaths caused by hepatitis B virus, and recombinant insulin is the basis for the treatment of diabetes. Nonetheless, there is still a need to expand the benefits of biopharmaceuticals use. For instance, poor vaccination coverage results in millions of deaths every year, mainly due to the lack of economic resources for vaccine production, necessary infrastructure, and personnel to properly accomplish vaccine delivery. Similarly, monoclonal antibodies and enzymes constitute the basis of therapies for a myriad of diseases, but their cost still limits their use [1]. Thus, it is crucial to develop bioprocesses that produce low-cost biopharmaceuticals as formulations that are easy and safe to administer, especially in developing countries [2]. Oral delivery of biopharmaceuticals is also proposed as an alternative with particular advantages, especially in the case of enteric pathologies where local action of the drug is required [3]. Therapies intended to induce **immunological tolerance** are also good candidates since the immune system associated to the intestine is of a highly tolerogenic nature [4].

These needs have inspired the development of innovative biopharmaceuticals production platforms that use organisms whose biomass is economically produced. This is the case of **molecular pharming**, which comprises the production of valuable molecules in genetically engineered plants. Since many plant species are edible, they are safe for oral administration, so the recombinant host serves as both the biopharmaceutical **biofactory** and the delivery vehicle [5]. Among such approaches, a technology that generated great expectations at the beginning of the 1990s was the concept of using genetically engineered plants as biofactories and delivery vehicles of biopharmaceuticals, primarily vaccines. This technology offers (i) low production costs, (ii) easy scale-up, (iii) high safety due to the lack of human pathogen replication in plants,

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(iv) high biosynthetic capacity, and (v) **bioencapsulation** effects, which are of special importance for an oral delivery that ensures the **bioavailability** of the therapeutic component [6].

During the 1990s and mid 2000s, the race for developing plant-based oral vaccines resulted in the evaluation of four candidates in clinical trials, and although these developments seem to be discontinued, they still revealed that plant-based vaccines have acceptable safety and also induce pathogen-specific immune responses following oral administration [7]. In the case of antibodies, hormones, and enzymes, the achievements of the last two decades include the expression of several candidates that proved to be functional. For instance, an anti-HIV antibody has been produced in a good manufacturing practice compliant (GMPc) process and the glucocerebrosidase enzyme is currently commercialized [8,9].

Although several reviews on the advances in the field of molecular pharming have been published over the last decade, the present review is focused on the recent achievements on the oral delivery of plant-made biopharmaceuticals, including vaccines, antibodies, and enzymes, among other therapeutics, which offer low costs and anticipate improvements in coverage and patient compliance.

Advances in Development of Plant-Based Oral Vaccines

The development of plant-based vaccines is an active research field (Table 1) with recent reviews reporting the state of the art [10,11]. The major advances in this field have been achieved using parenteral formulations. Some companies are conducting clinical trials using vaccines produced in *Nicotiana* plants, which transiently produce the antigen at high levels. After purification, the antigen is used to formulate injectable vaccines. This has been the case for vaccines against influenza, which are based on virus-like particles (VLPs) and are currently under clinical evaluations [12,13]. These vaccines are expected to be the first plant-made vaccines to be marketed. Other targets under study include rotavirus and rabies virus (http://www.medicago.com/English/Products/product-pipeline/default.aspx).

Development of oral vaccines imposes the challenge of accounting for suitable antigen formulation and immunization protocols in order to ensure antigen stability throughout the gastrointestinal tract, along with a sufficient immunogenic activity to surpass the tolerogenic nature of the **gut-associated lymphoid tissues** [14,15]. Therefore, the design of therapeutic proteins, and biopharmaceutical formulation; details of processing including freeze-drying, accompanying processing, and pharmaceutical form; and delivery regimen are critical aspects to ensure proper efficacy upon oral administration. In the case of vaccines, it is critical to achieve an efficient antigen uptake and presentation to T and B cells, which is indispensable for triggering robust adaptive immune responses that lead to desirable prophylactic/therapeutic effects (Figure 1). Many authors have focused on the following approaches: (i) the use of VLPs, which are protein complexes that self-assemble in the plant cell and render highly immunogenic complexes since they resemble viruses [16]; and (ii) the use of the B subunits of either cholera toxin or heat labile enterotoxin from *Escherichia coli* that act as **transmucosal carriers** due to their intrinsic property of efficiently crossing epithelial cells, thus exerting adjuvant effects to coadministered or fused antigens [17]. These antigen design approaches have led to the most promising findings in terms of oral immunogenic activity.

Other challenges faced in developing plant-oral vaccines include poor antigen yields and the lack of a proper dosing strategy. Freeze-drying was adopted later as an approach to stabilize plant biomass, concentrate the antigen, and achieve an accurate dosage by quantifying the antigen in terms of dry biomass weight [18]. Expression efficiency has also been optimized

Glossary

Bioaccesibility: the portion of a substance that can interact and be absorbed by an organism.
Bioavailability: a measure of the portion of a substance that, after administration, reaches the compartment in which it exerts its pharmacological activity.
Bioencapsulation: an effect mediated by a cell expressing biopharmaceuticals, which thanks to its structure, acts as a biological capsule that protects from degradations and delivers the target biopharmaceutical.
Biofactory: an organism or cell used to produce high value compounds, generally though genetic engineering.
Biopharmaceuticals: highly complex therapeutic molecules with diverse structures and are produced through genetic engineering approaches in several host organisms.
Gut-associated lymphoid tissues: a system of cells that mediates antigen sampling and the induction of adaptive immune responses after immunization through the oral route, which mainly comprises local IgA secretory responses. In addition, systemic, cellular and humoral responses can also be triggered.
Molecular pharming: technology focused on the production of biopharmaceuticals using plant biotechnology. This approach emerged in the early 1990s as an alternative to fermentation technology that offers high quality, low cost, enhanced safety, and avoidance of scale-up limitations.
Immunological tolerance: the lack of an immune response against components that would normally induce an immune response.
Plant cell wall: a structural organelle found in plant cells, mainly composed of cellulose, pectin, hemicellulose, and proteins.
Prime–boost schemes: immunization strategies in which an antigen is presented in different stages: priming, to induce primary immune response, and boosting, to mature and enhance the immune response. These schemes are used to achieve proper immune responses in terms of magnitude and polarization that lead to efficacious therapeutic or prophylactic effects.

through seed expression approaches, transplastomic technologies, and viral-vector-based systems [19].

Transmucosal carrier: molecule that possesses an intrinsic ability to cross the epithelial barrier, and also can be used to transport unrelated, genetically fused molecules through the mucosa, which favor immunogenicity.

Despite the accomplished advances, several aspects remain to be explored and validated in this field, including the optimization and characterization of distinct pharmaceutical dosage forms, assessment of long-term stability, and conducting phase II and III clinical trials. After more than two decades of investigations, the commercialization of oral plant-based vaccines has not become a reality and some authors discourage the idea due to their complexity and the lack of a well-defined regulatory framework for such vaccines. In contrast, others consider that, based on the outcomes from previous preclinical evaluations, plant-based vaccines can at least be realistically used in **prime–boost schemes** comprising parenteral priming with conventional vaccines or a plant-made purified antigen, and boosting with an oral plant-made formulation obtained by minimal processing [20]. At the preclinical level, some recently developed candidates proved to be immunogenic and immunoprotective in test mice upon oral administration. These include maize expressing hepatitis B surface antigen (HBsAg), used as a boosting agent [21], *Arabidopsis* expressing hemagglutinin H5 from the highly pathogenic avian influenza virus [22], and Indian mustard expressing the anthrax protective antigen [23]. Some authors have found that low doses of oral plant-based vaccine induced better humoral responses in mice when compared to high doses, including *Arabidopsis thaliana* and *Daucus carota* expressing the HIV-1 p24 antigen (used for priming) [24] and lettuce expressing the small HBsAg [25]. Unfortunately, no immunoprotection data have been provided for these candidates and translating these evaluations to larger animal models is a pending objective.

Other promising candidates include a vaccine targeting hepatitis C virus, consisting of a heterodimeric protein produced in tobacco plants, which proved to be orally immunogenic in mice when administered in a scheme comprising intramuscular injections of the same antigen produced in mammalian cells, leading to an increase in both mucosal and systemic immune responses [26]. Another study also used tobacco plants to express a self-adjuvating protein complex comprised of a dengue glycoprotein sequence and an immunoglobulin polymeric scaffold, which, following a subcutaneous immunization scheme, proved to be significantly immunogenic in mice after measuring both humoral and cellular responses [27]. An oral plant-based vaccine against measles has been evaluated in mice with interesting findings when applied in a prime–boost scheme combined with a DNA vaccine. Mice primed with the DNA vaccine and subsequently orally boosted with lettuce freeze-dried leaves expressing M antigen showed a tenfold increase in antigen-specific IgG titers [28].

A recent development in this field involved the use of transplastomic tobacco plants to produce a polio vaccine based on the VP1 protein, as an approach to achieve the induction of efficient mucosal immunity, which is not induced by the inactivated polio vaccines (IPVs) that are currently used. The plant-based VP1 vaccine administered with saponin and squalene as adjuvants provides short-term oral boosting in subcutaneously primed mice with the IPV, in terms of inducing highly specific mucosal (IgAs) and systemic antibody immune responses as well as neutralizing titers [29]. A subsequent study provided evidence on the effect of monthly oral boosts with the plant-based vaccine (with a 6-month gap without any boosting), followed by a single boost 1 year after priming [30]. Unlike the IPV, boosting with the plant-made VP1 vaccine allowed the development of a mucosal immune response and the induction of efficient high level and long lasting VP1-IgG1, IgA, and neutralizing antibody titers. The plant-made vaccine conferred the same level of protection against all three serotypes throughout the duration of this study whereas the IPV (single dose) led to absent or minimal levels of neutralizing

Table 1. Recent Key Advances in the Development of Plant-Based Oral Vaccines

Vaccine candidate	Adjuvants	Animal model	Immunization	Main findings	Refs
Poliovirus CTB-VP1 antigen produced in tobacco	Cholera toxin B subunit (CTB), saponin and/or squalene	Female CD-1 mice	s.c. priming with inactivated polio vaccine (IPV) 8 weekly oral boosts with CTB–VP1	Oral boosting after priming increased IgG1 and IgA titers when compared to IPV injections Observed seropositivity and neutralizing antibody titers for three virus serotypes when oral boosters are used	[29]
	CTB, saponin and/or squalene	CD-1 mice	s.c. priming with IPV 8 weekly oral boosts with CTB–VP1 followed by 3 monthly boosts Additional 2-monthly boosts after 6 months	VP1 with adjuvants induced high level and long-lasting significant IgG1 and IgA antibody titers from 29 to 400 days Conferred protection against three serotypes	[30]
Hemophilia A tolerogenic vaccine expressing coagulation factor (FVIII) antigens, heavy chain (HC) and C2, fused to CTB; produced in tobacco.	CTB	Male mice with hemophilia A and targeted deletion of *F8* exon 16 ($F8e16^{-/-}$) on a mixed C57BL6/129	CTB-HC and CTB-C2 mixture administered twice per week for 2 months Encapsulated FVIII concentrate was given i.v. weekly for at least 1 month	Confirmed delivery of bioencapsulated FVIII to the gut immune system Feeding of HC/C2 mixture significantly reduced T helper response and inhibitor formation against FVIII Prolonged oral delivery induced a long-term absence of inhibitor formation	[32]
Bioencapsulated Factor IX (F.IX) fused to CTB, acting as an hemophilia B tolerogenic vaccine; produced in tobacco	CTB	Male C3H/HeJ mice with targeted deletion of the F9 gene	Oral doses of CTB–FIX leaf material via oral gavage twice per week for 2 months Human FIX was given i.v. once per week after 1 month for 2 months	Feeding of CTB–FIX systemically delivers FIX Effectively blocks the formation of inhibitory antibodies Eliminated fatal anaphylactic reactions that occurred after four to six exposures to intravenous FIX	[33]
Acid α glucosidase (GAA) fused to CTB as a tolerogenic treatment for Pompe disease; produced in tobacco	CTB	Pompe mice (129SVE $Gaa^{-/-}$ mice)	Oral doses of gavage twice a week for 2 months i.v. injections of recombinant GAA weekly during the second month	Significant suppression of GAA specific IgG1 and IgG2 Lyophilization increased CTB–GAA concentration by 30-fold	[35]
Hemophilia B tolerogenic CTB-FIX fusion produced in lettuce	CTB	Hemophilia B dogs of the UNC-Chapel Hill strain	Bioencapsulated CTB–FIX was fed twice per week for 13 weeks	Significant suppression of IgG and IgE formation against intravenous FIX No side effects after feeding lyophilized CTB–FIX for >300 days	[34]
EspA vaccine candidate against enterohemorrhagic *Escherichia coli* (EHEC), produced transiently in *Nicotiana benthamiana* and transplastomically in *Nicotiana tabacum*	None	Canadian Arcott sheep (wethers)	Encapsulated vaccine was administered right before feeding through a cannula on days 1, 7, and 28 Wethers were challenged with *E. coli* O157:H7	Transgenic EspA has the potential to reduce EHEC shedding Intake of tobacco had no adverse effects on feed intake or health Particle size and time of immunization affect the residence of the vaccine in the rumen	[52]

Table 1. (continued)

Vaccine candidate	Adjuvants	Animal model	Immunization	Main findings	Refs
Cryj1 and Cryj2 antigens as vaccines against Japanese cedar pollen allergy produced in rice	None	Antigen-sensitized mice	Transgenic seeds were fed daily for 20 days After 2 weeks, Japanese cedar pollen was administered i.p. as a booster	Supression of allergen-specific $CD4^+$ cells Significant reduction of IgG and IgE Clinical symptoms were reduced in nasal tissue	[53]
	None	BALB/c mice	Mice were fed once a day for 20 days in conjunctivitis prevention model and 16 days in established conjunctivitis model Mice were sensitized with two i.p. injections of pollen plus alum Challenged with pollen in eye drops	Eosinophils, inflammatory cells and clinical were significantly reduced Serum concentration of IgE was significantly lower Significant reduction of cytokines secreted by splenocytes Significant increase in the production of interferon-γ by splenocytes	[54]

Abbreviations: i.p., intraperitoneal; i.v., intravenous; s.c., subcutaneous.

antibodies against serotypes 1 and 2 but also resulted in neutralizing antibodies against serotype 3.

Tolerogenic vaccines deserve special consideration for allergies or autoimmune diseases. This therapeutic approach takes advantage of the tolerogenic nature of the gut-associated immune system [15]. At the preclinical level, the current developments have proven the concept of tolerance induction against allergies (e.g., mite allergy) using rice-based vaccines in mice [31]. Also, the induction of tolerance to prevent the production of blocking antibodies against replacement therapy proteins has been achieved in mouse models. For instance, hemophilia is a congenital bleeding disorder in which the functional factors VIII or IX are not produced, so the therapy consists of administering either recombinant factor VIII or factor IX. To prevent the formation of antibodies against factors VIII/IX, they have been targeted through orally administered plant-based vaccines to induce tolerance in a mouse model [32,33].

Herzog and colleagues [34] opted to use the hemophilia B dog model, comparable in size to pediatric patients, as a novel approach to test this kind of therapy, as data from non-mouse models are scarce. The B subunit of cholera toxin (CTB), which acts as a transmucosal/immunomodulatory protein carrier, was fused to human coagulation factor IX (FIX) and genetically transferred into chloroplasts to be expressed in lettuce plants (Table 2); tobacco does not have the generally recognized as safe (GRAS) status, which limits its clinical application. The results for the bioencapsulated and orally administered CTB–FIX scheme supported that oral tolerance is attainable by administering the tolerogenic treatment based in transplastomic lettuce, avoiding the formation of pathogenic antibodies in large non-mouse animal models. Boxes 1 and 2 present the cases of plant-made oral vaccines characterized in detail. Pompe disease, a rare muscular disease caused by the deficiency of the enzyme acid α-glucosidase, is treated by administering a recombinant version of the enzyme. Similarly, in an effort to develop approaches to prevent the formation of antibodies against the therapeutic enzyme, a tolerogenic plant-based vaccine candidate against this target was reported [35].

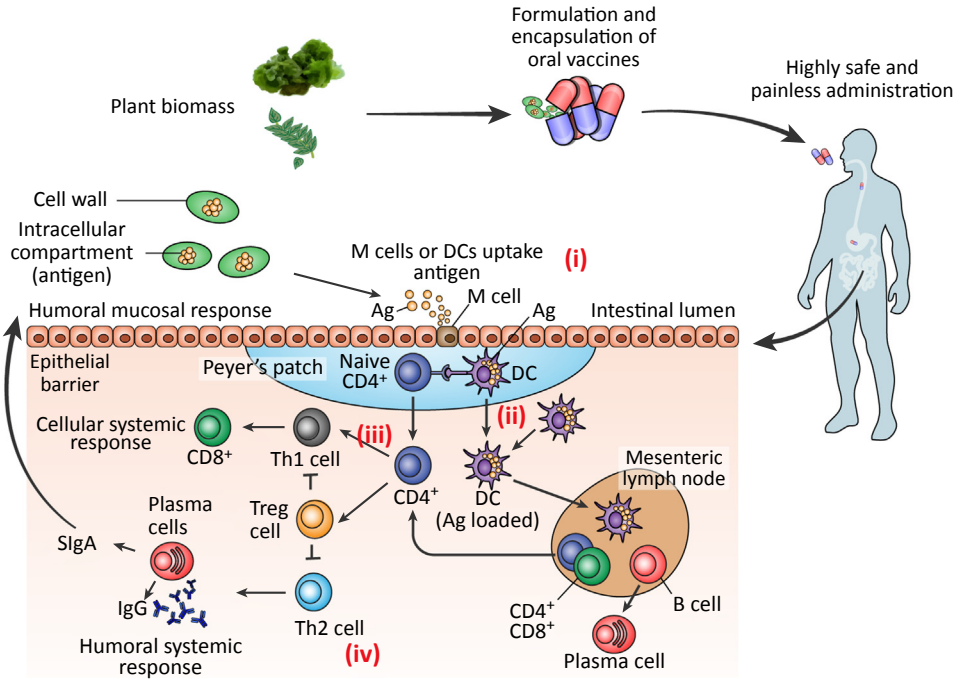

Figure 1. Action Mechanisms for Plant-Based Oral Vaccines. Raw plant tissues that express the desired protective antigen are processed and stabilized, through methods such as lyophilization, to create a biomass that is easily formulated as oral vaccine. Following oral administration, the plant cell biocapsules achieve a balance between protecting the antigen from degradation during its transit through the gastrointestinal tract and releasing the antigen, thus the antigen becomes accessible to the gut-associated lymphoid tissues (i). The proper release of the antigen allows its uptake by M cells or DCs. Antigen-presenting cells (e.g., DCs) recognize the antigen through pattern recognition receptors (e.g., Toll-like receptors) and process it into short peptides (epitopes) that are presented through MHC-I or MHC-II molecules to the lymphocytes located at the Peyer's patch or in the mesenteric lymph node (ii); MHC-II molecules mediate epitope presentation to $CD4^+$ cells that according to several conditions will support the induction of cytotoxic T lymphocyte responses (mediated by MHC-I) (iii) and/or humoral responses mediated by plasma cells (iv). Abbreviations; Ag, antigen; DC, dendritic cell; M, microfold; SIgA, secretory IgA; Th1, T helper 1; Th2, T helper 2; Treg cell, T regulatory cell.

The activity of oral plant-based vaccines and even the apparently enhanced immunogenicity of these plant-based formulations when compared to conventional vaccines raised interesting questions regarding the involved biological mechanisms behind this activity. The role of plant cell components in promoting vaccine activity has been hypothesized but not systematically studied and constitutes a relevant path for research [14]. The cases cited in this section indicate how active is the research field on orally delivered vaccines and reinforce the hope to address the pending objectives to make oral plant-based vaccines a reality.

Advances in Plant-Based Oral Immunotherapies

Biopharmaceuticals Administered through a CTB-Based Delivery Approach

Recent reports have shown the results of oral delivery of plant-made biopharmaceuticals in murine models. The delivery approach relies on the use of CTB as the transmucosal carrier since this molecule binds the GM1 ganglioside, which is present in gut epithelial cells, with subsequent translocation of the genetically fused biopharmaceutical to the submucosa, where it becomes accessible to the systemic compartment and can reach distant tissues. The approach consisted of fusing the biopharmaceutical to CTB with furin cleavage sites placed in between to mediate

Table 2. Compilation of Biopharmaceuticals Orally Delivered in Mice through Transplastomic Plant Cells Using a CTB-Based Approach

Biopharmaceutical	Application	Mice model	Main findings	Refs
Proinsulin expressed in tobacco and lettuce	Treatment of type 1 diabetes	C57BL/6 mice	Reduced glucose levels	[36]
ACE2 and Ang-(1–7) expressed in tobacco	Treatment of inflammatory disorders and PH	C57Bl/6J mice with EIU B10.RIII mice with autoantigen-induced experimental autoimmune uveoretinitis (EAU) Sprague–Dawley rats with induced PH	Reduced both EIU and cellular infiltration, retinal vasculitis, damage and folding in EAU Reduced PH in both prophylactic and therapeutic models	[37,38]
MBP expressed in tobacco	Treatment of Alzheimer's disease	Transgenic Alzheimer's disease (3 × TgAD) mice	Increased the levels of MBP in the brain, reduced amyloid levels in the hippocampus and cortex brain region; and reduced amyloid loads *ex vivo*. Reduced Aβ42 in the retina and prevented loss of retinal ganglion cells.	[39]
Exendin-4 expressed in tobacco	Treatment of type 2 diabetes	C57BL/6 mice with spike blood glucose levels	Induced insulin secretion, reduced glucose levels but avoiding hypoglycemia	[40]

the release of the therapeutic (Figure 2). The expression approach is based on transplastomic technologies that allow an efficient accumulation of the recombinant proteins in the chloroplasts, and oral administration in mouse models has been performed to evaluate the potential of the therapies. This concept has been applied to a few cases. In insulin, it reduces serum glucose levels at a comparable magnitude to that of commercial insulin upon treatment with homogenized fresh leaf tissues [36]. In angiotensin-converting enzyme (ACE)2 and angiotensin (Ang)-(1–7), it reduced ocular inflammation in mouse models of endotoxin-induced uveitis (EIU) and autoantigen-induced experimental autoimmune uveoretinitis, which were fed with either fresh or freeze-dried plant cells [37]; and reduced pulmonary hypertension (PH) in a model of male Sprague–Dawley rats, with improved associated cardiopulmonary pathophysiology in a PH prevention protocol. In the same model, this approach arrested disease progression, improved right heart function, and decreased pulmonary vessel wall thickness in a reversal protocol. The combination of ACE2 and Ang-(1–7) was also assessed, resulting in multiple beneficial effects against PH-induced lung damage [38]. In myelin basic protein (MBP), which increased the levels of MBP across the mice brain upon oral administration of lyophilized cells, this approach reduced amyloid loads in both human and mouse brains after CTB–MBP incubation *ex vivo*, reduced amyloid levels in the hippocampus and cortex brain region, reduced amyloid β (Aβ)42 in mouse retina, and prevented the loss of retinal ganglion cells in transgenic Alzheimer's disease mice (3 × TgAD) [39]. Administration of exendin-4 in the form of lyophilized cells decreased glucose levels in mice while avoiding hypoglycemia in a model of mice with spiked blood glucose levels [40].

Box 1. A Case of an Oral Vaccine for Veterinary Use

A model of an orally administered vaccine against EHEC for ruminants has been reported by Miletic and colleagues [52]. The authors assessed the expression of a set of vaccine candidate genes from EHEC: *EspA*, *EspD*, *Tir*, *NleA* and the B subunit from the Shiga toxin, *Stx2b*. Five recombinant proteins were designed as vaccine candidates and expressed transiently in *Nicotiana benthamiana* and transplastomically in *Nicotiana tabacum*. Three of these EHEC proteins, NleA, Stx2b, and a fusion of EspA accumulated when transiently expressed. Transient protein accumulation peaked when EHEC proteins were fused to an elastin-like polypeptide (ELP) tag.

The authors decided to focus the study on the ESPA antigen, which was successfully expressed through transplastomic technologies, with yields of up to 479 mg/kg leaf material dry weight. The plant-made formulation was administered to wethers (5 or 10 mg doses). The authors controlled the particle size by grounding plant tissue and passing the material through a 4-mm screen and then packing the resulting powder into gelatin capsules. The vaccine was administered immediately before feeding through a cannula on days 1, 7, and 28. Sheep were subsequently challenged with *E. coli* O157:H7. These results suggest that plant-made, transgenic EspA has the potential to reduce EHEC shedding in ruminants. Neither wild-type tobacco nor transformed tobacco had adverse effects on feed intake or health of the wethers.

The authors also assessed the stability of the recombinant EspA protein in the rumen and in the digestive tract by collecting samples from the tissues of the immunized animals. The intact antigen was detected in the rumen up to 6 h after administration; whereas fragments were detected up to 24 h, which supports the idea that the plant cell protects the antigen from degradation in the rumen. Doubling the dosage of EspA also doubled the time that EspA remained intact in the rumen, which highlights the importance of optimizing antigen dosage for oral administration. Furthermore, the administered leaf material was of a particle size that would rapidly pass from the rumen, limiting the extent of microbial digestion in the rumen. The authors also attribute the success of vaccination to the fact that wethers were fed a pelleted, grain-based diet to decrease the residence time of the vaccine in the rumen. This study constitutes a relevant precedent that emphasizes critical aspects to be considered in the optimization of plant-based oral vaccines, including particle size, time of immunization with respect to feeding and dosage.

Antihypertensive Peptides

Novokinin is a peptide (sequence: RPLKPW) derived from ovalbumin that, according to empirical evidence, can be used as an orally administered fusion protein for hypertension treatment due to its vasodilating activity. Wakasa and colleagues [41] designed a protein comprising ten or 18 tandem repeats of Novokinin and an endoplasmic-reticulum-retention

Box 2. The Case of Plant-Made Oral Vaccines against Pollen Allergy

A group from Japan has developed oral vaccines against Japanese cedar pollen allergy. The vaccine consists of rice expressing modified versions of the Japanese cedar pollen antigens, namely Cryj1, which was obtained by sequence fragmentation, and Cryj2 obtained by molecular shuffling. The fragments of the former are expressed as fusion proteins based on rice seed storage glutelins, whereas the latter is expressed individually. Under this approach T cell, but not B cell, epitopes are retained and thus the antigen is not recognized by pollen antigen-specific IgE but is able to induce tolerogenic responses. The Cry antigens are accumulated in endoplasmic-reticulum-derived protein bodies that are convenient antigen delivery vehicles as they are highly resistant to the extreme conditions within the gastrointestinal tract [53].

Following oral immunization schemes, this vaccine has been shown to prevent the development of allergic conjunctivitis in mice [54]. However, the goal of allergy therapies is to achieve treatments that efficiently alleviate the manifested allergy. Therefore, in a further study the authors assessed the ability of the rice-based oral vaccine to ameliorate established allergic conjunctivitis in mice [55]. In such trials the animals were sensitized by immunizing the mice twice through intraperitoneal injection with Japanese cedar pollen plus alum, subsequently challenged with pollen in eye-drops, and finally fed for 16 days with transgenic rice seeds expressing modified Japanese cedar pollen allergens Cryj1 and Cryj2 or nontransgenic rice seeds. The mice were finally challenged with pollen in eyedrops. Interestingly, the rice-based tolerogenic vaccine induced a decrease on the number of eosinophils in the conjunctiva and the clinical score for conjunctivitis as well as an increase in the production of interferon-γ by splenocytes. The vaccine had no effect on the $CD4^+CD25^+Foxp3^+$ regulatory T cell population in the spleen or submandibular or mesenteric lymph nodes. Although detailed characterization of the immune mechanisms explaining the effects of this vaccine remains to be completed, the present evidence allows the authors to postulate this approach as an attractive and potential treatment for pollen allergy.

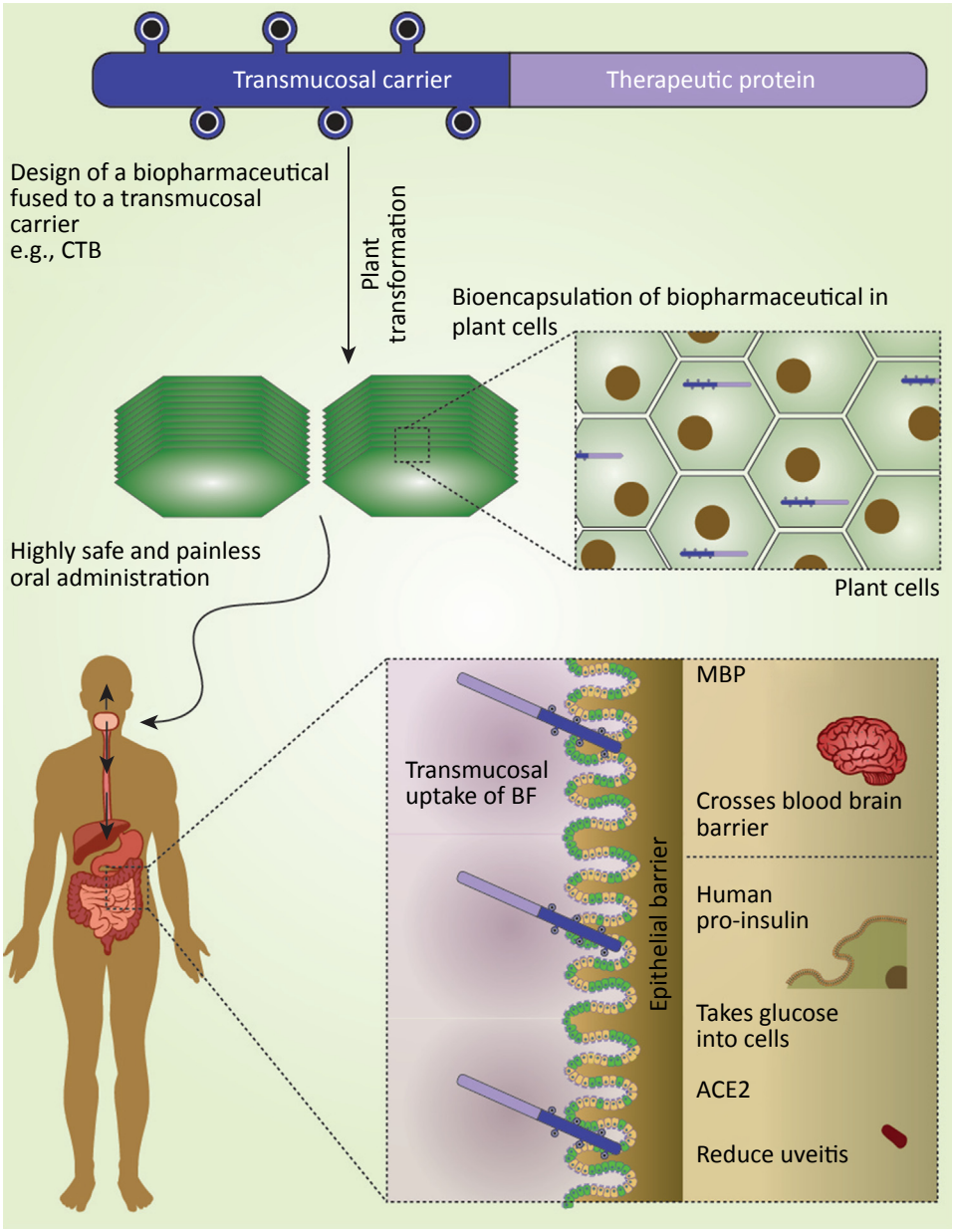

Figure 2. Design, Production and Delivery of Oral Biopharmaceuticals by Plant Cells. The diagram illustrates an example of how a gene that codes for a target biopharmaceutical is designed to drive the expression of a fusion protein that comprises the biopharmaceutical of interest (e.g., enzymes and hormones) and a transmucosal carrier, such as CTB, with furin cleavage sites in between. Genetic transformation approaches allow the transfer of the transgene into the plant cell to develop biopharmaceutical–producer plants or cell lines. Plant cells act as biocapsules that are stable upon lyophilization and efficiently deliver the biopharmaceutical upon oral administration. During transit through the gastrointestinal tract, plant biomass releases the therapeutic fusion protein and the CTB carrier mediates the binding of the molecule to the gangliosides on the surface of epithelial cells with its subsequent translocation to the submucosa. Furin cleavage sites mediate the release of the biopharmaceutical in the systemic compartment, so it can reach systemic targets or even distant target tissues. This concept has been proven for the delivery of several biopharmaceuticals (see Table 2), including insulin that was able to reduce blood glucose levels and MBP able to reach the central nervous system and retina, reducing amyloid levels. Abbreviations: ACE2, angiotensin-converting enzyme 2; BF, biopharmaceutical; CTB, cholera toxin B subunit; MBP, myelin basic protein.

signal (amino acid sequence: KDEL) at the C terminal, which was expressed at the nuclear level in rice seeds (cultivar Kita-ake) through *Agrobacterium*-mediated transformation. The protein carrying Novokinin was deposited in the nucleolus of the endosperm cells. Seeds containing the recombinant protein were orally administered to spontaneously hypertensive rats to evaluate the possible reduction of systolic blood pressure, observing a more positive antihypertensive activity when compared to previous studies in which Novokinin was expressed as a fusion protein.

Antibodies and Blocking Agents

The delivery of *Arabidopsis*-made monoclonal antibodies that protect against enterotoxigenic *Escherichia coli* (ETEC) infection has been explored by Virdi and colleagues [42]. Four ETEC variable domains of llama heavy chain-only antibodies (VHHs) bearing F4 fimbriae (anti-$F4^+$ ETEC), capable of retaining their functionality in harsh environmental conditions, were fused to the crystallizable region (Fc region) fragment of porcine immunoglobulin (IgG or IgA) to promote agglutination of ETEC and prevent its attachment to the gut. The constructed antibodies were expressed in *Arabidopsis* seeds and orally administered to weaned piglets. Analysis of the feces of VHH-IgA fed piglets showed a decline in bacteria shedding and lower serological reaction to ETEC, which indicated lower exposure to this pathogen. Unfortunately, the VHH-IgG group failed to show an effective response.

Ilan and colleagues [43] reported an approach to treat inflammatory disorders through an oral immunotherapy delivered by plant cells. The authors assessed in mice the immunomodulatory effects of an orally administered recombinant anti-tumor necrosis factor (TNF) fusion protein (called PRX-106), which was expressed in BY-2 plant cells (*Nicotiana tabacum*) and consisted of the soluble form of the human TNF receptor (TNFR) fused to the Fc component of a human IgG1 domain. Hepatitis and colitis mouse models were used to compare the activity of the plant-mediated delivery versus a commercially available recombinant TNFR fusion protein (called etanercept) administered through a parenteral scheme. Results showed that the oral administration of lyophilized BY-2 cells changed the immune balance of the mice by increasing their anti-inflammatory capabilities, which manifested through improved condition of the liver (hepatitis model), the alleviation of body weight decrease, and improvement of damaged bowels (colitis model). Moreover, a general increase of systemic immune responses regulated by the reduction of proinflammatory cytokines in the hepatitis model as well as an increase of anti-inflammatory cytokines and regulatory T cells in the colitis model was observed. These results were deemed favorable compared with those previously obtained by studies of Crohn's disease patients treated with parenterally administered anti-TNF compounds such as etanercept, which showed a lack of immunomodulatory effects at the level of the gut-associated lymphoid system [44]. PRX-106 has been evaluated in a phase I clinical trial to determine its safety and the immunomodulatory effect upon oral administration, assessing three different doses (2, 8 or 16 mg/day) administered for five consecutive days in 14 healthy volunteers [45]. The treatments were found to be safe and well tolerated with no significant absorption. All the tested doses induced an increase in $CD4^+CD25^+$ and $CD8^+CD25^+$ subset of suppressor lymphocytes. The group that received the 8 mg dose showed a marked increase in $CD4^+CD25^+FoxP3^+$ regulatory T cells. In addition, natural killer T regulatory cells, $CD3^+CD69^+$ and $CD4^+CD62^+$ lymphocyte subsets increased with treatment. No changes in serum TNFα were observed. Therefore, this plant-based oral immunotherapy is proposed as a promising approach to treat autoimmune, TNF-mediated diseases, such as inflammatory bowel diseases and nonalcoholic steatohepatitis.

Therapeutic Enzymes

A recent study reported that β-glucocerebrosidase (approved for human use in a parenteral formulation) is successfully delivered by the oral route when carrot cells are used as delivery vehicles in test rats and pigs [46]. This report has generated great expectations in the area of oral immunotherapies based on plant-made biopharmaceuticals. As of today, the only plant-made biopharmaceutical that has been approved by the FDA and commercialized is ELELYSO, a parenterally delivered drug consisting of the glucocerebrosidase enzyme used for replacement therapy against Gaucher's disease [47]. In this case, the regulatory approval was facilitated due to the production within a contained environment that offers high biosafety and is similar to the conventional fermentation systems; facilitating gene containment and implementation of a GMPc process. This way, a large pharmaceutical company, Pfizer, commercialized this biological drug produced in plant cells. This case gained attention due to the attractive biosynthetic capacity of plant cells for this particular enzyme and problems of viral contamination in the conventional production platform [48].

Concluding Remarks and Perspectives

Current evidence suggests that the plant cell acts as a capsule that efficiently protects biopharmaceuticals from degradation in the gastrointestinal tract and, at the same time, mediates their proper release in order to achieve a bioavailability that will ensure therapeutic effects. It is well established that the physical encapsulation of intracellular nutrients by cell walls plays a predominant role in influencing macronutrient bioaccessibility (release) from plant foods during human digestion. The knowledge gained in this field highlights that using different processing protocols of plant material may affect the integrity of the cell walls and thus influence the bioavailability/stability balance of a particular formulation. Studies evaluating these aspects are needed to characterize and optimize the plant-based oral formulations intended to deliver biopharmaceuticals by the oral route (Box 3).

The proof of concept for the oral delivery of biopharmaceuticals, using CTB as a transmucosal carrier, to the local intestinal environment, the systemic compartment, or even distant tissues beyond the blood–brain barrier, brings new hope to this field. However, the replication of such studies by more research groups, especially those evaluating the delivery of therapeutics in distant tissues, will strengthen the evidence and will encourage the application of such approaches in the development of therapies for other pathologies. An important aspect to be defined is the safety implications of using CTB as the biopharmaceutical translocator because this molecule possesses immunomodulatory activity that may lead to undesired effects.

Vaccine development stands as the major challenge in this field since the induction of immunoprotective responses depends on several factors and candidates should be evaluated in a large population and thus phase III clinical trials and several other regulatory paths should be addressed. In this regard, the achievements on the clinical use of the first plant-made biopharmaceutical constitute a critical advance opening a facilitated path for the approval of plant-based vaccines. Although some phase I clinical trials evaluating oral plant-based vaccines were conducted, the last one was reported 10 years ago, and these specific developments were not continued. However, the advances in the commercialization of plant-made biopharmaceuticals, improvements in plant expression systems, and efforts to account for a regulatory framework have generated new hopes for this field and thus important perspectives have risen for the following years.

Outstanding Questions

Will plant-based strategies for the oral delivery of biopharmaceuticals mature to be evaluated in clinical trials?

Is it possible to develop efficient oral formulations based on plant cells that can serve as prophylactic or therapeutic interventions against human diseases?

Is it possible to characterize the mechanisms of release of biopharmaceuticals by plant cells to rationally design plant-based formulations?

Will the industry and regulatory approval systems adopt plant-based systems to innovate the field of biopharmaceuticals production?

Box 3. Bioencapsulation Exerted by the Plant Cell

Understanding in detail how the plant cell acts as a biocapsule that delivers biopharmaceuticals to the gut remains a pending objective. However, several aspects can be discussed and hypothesized based on the current knowledge on food digestion, especially fiber. In fact, unbroken cell walls present in foods act as capsules for the intra-cellular components, thus regulating their **bioaccesibility** and breakdown by digestive enzymes [56]. Some authors have suggested that the release of the biopharmaceutical only occurs after the action of bacterial enzymes produced by gut microflora due to the fact that the **plant cell wall**, which is prominently constituted by cellulose, cannot be degraded by human digestive enzymes (Bacteroidetes and Firmicutes) [57]. In fact, the cell wall in dicotyledonous plants is mainly composed of non-starch polysaccharides that primarily include, in nonlignified tissues, the following: (i) cellulose (30%), (ii) hemicelluloses (30%), and (iii) pectin (35%), which are not digested by human digestive enzymes. However, gut microflora produces enzymes that lead to the digestion of such polymers, mainly in the large intestine [58,59]. Nonetheless, it should be considered that the interaction of a plant biomass-based formulation with the digestive system is complex and comprises a number of events that may explain how the biopharmaceutical is protected from degradation while it also becomes available for absorption. For instance, physical breaking of the plant cell is a phenomenon that may result in cells with compromised cell wall integrity, which allows the release of the biopharmaceutical. In the nutrition field, it is well known that after mastication, food material enters the stomach through the esophagus. In the antrum of the stomach, mechanical and chemical processes facilitate further breakdown where particles are formed with a surface area that allows the penetration of enzymes and acids that are essential for digestion [56]. Therefore, the release of biopharmaceuticals is not only dependent on cell wall enzymatic degradation. Physical breaking should be especially considered in the case of formulations made with freeze-dried, pulverized material, which alters cell wall integrity. Surprisingly, detailed studies on how plant biomass processing modifies the biopharmaceutical release efficiency have not been conducted. We consider that the release of the biopharmaceutical should not be explained only in terms of cellulose as the unique component of the cell wall. Another aspect to consider are the effects caused by the digestion of cell wall proteins (accounting for the 5% of its structure) by digestive proteases. In addition, the release of soluble polysaccharides during the transit through the stomach and intestines should also be considered as a phenomenon that allows the release of intracellular components. We propose that the combination of these events explains the release of biopharmaceuticals in the gut and that future investigations will be critical in expanding the knowledge on this matter, opening the path for the optimization of plant-made biopharmaceutical formulations.

The structure and behavior of cell wall polysaccharides as well as the macroproperties of the cell wall matrix for particular plant species have an influence on the disassembly of plant foods and the release of nutrients during digestion [56]. Therefore, it is expected that important variations that exist among plant species in their cell wall permeability, susceptibility to physical disruption, or proportion of protein and soluble polysaccharides will result in a differential efficiency in the biopharmaceutical delivery.

Lyophilization of plant tissues is an attractive alternative to obtain oral formulations for biopharmaceuticals since this procedure allows the concentration of the biopharmaceutical as well as a long-term storage. In addition, some authors have paid attention to the reduction of the bacterial load caused by freeze-drying. However, in our opinion, this factor will not be critical considering that plant materials should be produced under GMPs and that oral vaccines do not require strict purification to decrease microbial contamination. Few studies on optimizing the freeze-drying process to ensure antigen stability have been reported. Czyż and colleagues [60] evaluated several parameters during lyophilization of lettuce leaves expressing HBsAg, reporting that the profile of 20°C (shelf temperature) for 20 h for primary and 22°C for 2 h for secondary drying lead to an efficient process. In addition, the use of sucrose allows efficient antigen stabilization. Other identified key aspects are freezing rate and postprocess residual moisture. Under this protocol, the author achieved yields of 200 μg/g dry weight of functional VLPs. Another aspect to consider is the particle size, which can affect the release and protection of the biopharmaceutical. Particle size is easily controlled by filtering the pulverized dry material through a mesh. This powder can be packaged in capsules or resuspended in saline for oral gavage. Machines are commercially available for such processing and packaging of capsules from lyophilized leaves. Particle size distribution, which reflects the extent of deformation and disintegration of a plant food, is an important parameter, as it affects the subsequent digestion processes including gastric emptying and sieving as well as digestibility in the small intestine. Particles of smaller size possess a larger proportion of fractured cells, and therefore exhibit greater losses for their encapsulated content. Furthermore, fissures running through the core of plant tissue particles can be created, as observed in food studies. To reach the small intestine through the duodenum, particles need to reach <1–2 mm in size, according to previous fiber related studies in humans [56].

Realistically, the development of oral plant-based vaccines still requires the accomplishment of several research steps. At the moment, one possibility consists of using plant-based formulations in the form of capsules made with freeze-dried plant material [49]. The development of oral vaccines is considered viable at least as boosting agents in prime–boost schemes and further advances in the

area will depend, in part, on suppressing the bottleneck of translating the results from initial tests in mice to larger animal models and subsequently to humans. The case of DNA vaccines illustrates the challenge of translating the promising findings found in murine models into humans [50].

The successful adoption of plant-based oral biopharmaceuticals will also require surpassing the 'valley of death' as occurs for any technology, which requires fulfilling the regulatory framework and finding a proper niche through investment from big pharmaceutical companies or international humanitarian organizations and governments. In conclusion, the road to having oral plant-based biopharmaceuticals available in the clinic is still long but recent advances have provided new hopes for the field (see Outstanding Questions).

Besides the regulatory aspects, the interest of companies to commercialize plant-based biopharmaceuticals adds another factor to the situation; unfortunately, it will mainly rely on economic interests and not on a humanitarian focus. This might be the reason for the lack of commercialization of a vaccine approved for veterinary use by the US Department of Agriculture [51]. Considering that low-cost biopharmaceuticals are urgently needed in developing countries, the humanitarian use of this technology is envisioned as the solution. Nonprofit institutes/academies and governments should play a pivotal role in this vision. However, only a few organizations are currently working to develop plant-based biopharmaceuticals under this philosophy.

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Disclaimer Statement

The authors declare no conflict of interest.

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