



An overview of tolerogenic immunotherapies based on plant-made antigens

Dania O. Govea Alonso, Jaime I. Arevalo-Villalobos, Verónica A. Márquez-Escobar, Sornkanok Vimolmangkang & Sergio Rosales-Mendoza

To cite this article: Dania O. Govea Alonso, Jaime I. Arevalo-Villalobos, Verónica A. Márquez-Escobar, Sornkanok Vimolmangkang & Sergio Rosales-Mendoza (2019): An overview of tolerogenic immunotherapies based on plant-made antigens, Expert Opinion on Biological Therapy, DOI: [10.1080/14712598.2019.1597048](https://doi.org/10.1080/14712598.2019.1597048)

To link to this article: <https://doi.org/10.1080/14712598.2019.1597048>



Accepted author version posted online: 20 Mar 2019.



Submit your article to this journal [↗](#)



Article views: 14



View Crossmark data [↗](#)

Publisher: Taylor & Francis

Journal: *Expert Opinion on Biological Therapy*

DOI: 10.1080/14712598.2019.1597048

An overview of tolerogenic immunotherapies based on plant-made antigens

Dania O. Govea Alonso^{1,2}, Jaime I. Arevalo-Villalobos^{1,2}, Verónica A. Márquez-Escobar^{1,2}, Sornkanok Vimolmangkang³, Sergio Rosales-Mendoza^{1,2*}

¹Laboratorio de Biofarmacéuticos Recombinantes, Facultad de Ciencias Químicas, Universidad Autónoma de San Luis Potosí, Av. Dr. Manuel Nava 6, San Luis Potosí, 78210, México; ²Sección de Biotecnología, Centro de Investigación en Ciencias de la Salud y Biomedicina, Universidad Autónoma de San Luis Potosí, Av. Sierra Leona 550, Lomas 2ª. Sección, San Luis Potosí, 78210, México.

³Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand.

*Author for correspondence

Sergio Rosales-Mendoza

Facultad de Ciencias Químicas, Universidad Autónoma de San Luis Potosí

Av. Dr. Manuel Nava 6, SLP, 78210, México

Phone: 444-826-2440

Fax: 444-826-2440

E-mail: rosales.s@uaslp.mx

Abstract

Introduction: Over the last two decades, genetically engineered plants became attractive and mature platforms for producing vaccines and other relevant biopharmaceuticals. Autoimmune and inflammatory disorders demand the availability of accessible treatments and one alternative therapy is based on therapeutic vaccines able to down-regulate immune responses that favor pathology progression.

Areas covered: The current status of plant-made tolerogenic vaccines is presented with emphasis on the candidates under evaluation in large test animals. Nowadays this concept has been assessed in models of food and pollen allergies, autoimmune diabetes, asthma, arthritis, and prevention of blocking antibodies induction against a biopharmaceutical used in replacement therapies.

Expert Opinion: According to the current evidence generated at the preclinical level, plant-made tolerogenic therapies are a promise to treat several immune-related conditions and the beginning of clinical trials is envisaged for the next decade. Advantages and limitations for this technology are discussed.

Keywords: oral tolerance, regulatory T cell, allergy, transgenic plant, asthma, diabetes, arthritis

GLOSSARY

Th2: Type 2 T helper cells

IgE: Immunoglobulin E

IgG1: immunoglobulin G isotype 1

Cry j 1: Japanese cedar pollen protein 1

Cry j 2: Japanese cedar pollen protein 2

GFP: green fluorescent protein

ER: endoplasmic reticulum

PB-I: protein body I

PB-II: protein body II

IL-4: interleukin 4

IL-5: interleukin 5

IL-13: interleukin 13

IL-10: interleukin 10

Chao 1: T cell epitope 1 from Japanese cypress pollen protein

Chao 2: T cell epitope 2 from Japanese cypress pollen protein

Cys: cysteine

F.VIII: Coagulation factor VIII

F.IX: Coagulation factor IX

hF.IX: Human coagulation factor IX

TGF- β : Transforming growth factor- β

LAP: Latency associated peptide

T1D: Type 1 diabetes

T1DM: Type 1 diabetes mellitus

IAA: autoantibodies to insulin

GADA/GAA: glutamic acid decarboxylase

GAD65: human glutamic acid decarboxylase

IA-2AA: tyrosine phosphatase IA2

NOD: non-obese diabetic

PLP: pyridoxal 5'-phosphate

CTB: cholera toxin B subunit

GM1: monosialotetrahexosylganglioside

Accepted Manuscript

1. Introduction

Modern biotechnology has accelerated the development of new vaccines and immunotherapies [1]. Innovative production platforms for recombinant antigen production can be exploited to diminish costs and expand the benefit of such therapies in developing countries especially. Plants offer a platform for the production of functional complex proteins using low-cost culture medium/substrates and can be even used as the delivery vehicle, which greatly decrease formulation and administration costs when compared to parenteral treatments [2]. Other attractive attributes of such vaccines include low cost, safety (no human pathogens are replicated in the host), and high biosynthetic capacity [3].

Since the pioneering studies performed by Curtis and Cardineau [4], the development of vaccines based on genetically engineered plants has become a well-accepted and matured platform. Improvements in yields [5, 6] and plant material processing, along with fulfillment of regulatory requirements [7], have been addressed after decades of research and development. Plant-made vaccines prototypes have been developed over the last three decades with some of them under clinical evaluation and production under GMPs [8-10]. The most representative achievements in the area of plant-made biopharmaceuticals comprise the case of glucocerebrosidase (currently commercialized for human use to treat Gaucher's disease) and the antibody cocktail to treat patients infected with the Ebola virus [11, 12].

An important group of diseases targeted with plant-based vaccines is the case of autoimmune and inflammatory disorders. These conditions demand the availability of accessible biopharmaceuticals to develop immunotherapies that rely on down-regulating the causative immune responses. Since the immune system associated to the gut is amenable for the induction of tolerance; oral plant-based vaccines are convenient to treat such diseases, in particular following schemes comprising high and repetitive doses and the use of specific immunomodulatory protein carriers [13].

The present review covers the current outlook on the development of plant-based immunotherapies for the treatment of autoimmune disorders and other conditions related to undesired immune responses.

2. Therapeutic implications of tolerogenic immune responses

Immune tolerance relies on complex physiological mechanisms that are dependent on several factors, including the dose and time of antigen exposure as well as the administration route. Genetics as well as composition and metabolic activity of the microbiome also exert an important influence on immune tolerance [14]. In particular oral tolerance is induced in the gut-associated lymphoid tissues (GALT), which comprise several different organized lymphoid structures that include Peyer's patches (PPs) and Isolated lymphoid tissue (ILF) being critical for the initiation of intestinal IgA responses [15]. The induction of tolerance primarily comprises the suppression of antigen-specific effector T and B cell responses by the induction of T cell deletion (anergy) and the activation of Treg cells [16].

Antigens are primarily taken up at the intestinal level by microfold (M) cells, epithelial cells, and dendritic cells (DCs) leading to antigen presentation to T cells, which is performed by DCs that in fact can directly capture soluble low molecular weight antigens from the intestinal lumen through dendrits. DCs are professional antigen-presenting cells that coordinate processes of innate and adaptive responses; they also contribute to the induction of peripheral tolerance. On the other hand enterocytes express MHC class I and II molecules, but not co-stimulatory molecules; thus they do not lead to T cell activation and in consequence act as tolerogenic antigen presenting cells that contribute to the development of oral tolerance [15, 17].

Among the different subsets of DCs in the lamina propria, CD11b⁺CX3CR1⁺ cells do not migrate to MLN but produce IL-10 [18]. In contrast, CD11c⁺CD103⁺CX3CR1⁺ cells are able to migrate to MLN [17] and present the antigen to naïve CD4 cells in a way that promotes their differentiation to regulatory T cells (Tregs) through a complex mechanism that involves the secretion of metabolites (*e.g.* retinoic acid), enzymes (*e.g.* indoleamine 2,3-dioxygenase and retinal dehydrogenase), and cytokines (*e.g.* TNF- β and IL-10) [19]. DCs from most tissues are typically activated upon PAMPs recognition from bacteria via TLRs. In contrast, since gut DCs are continuously exposed to gut microflora these often require additional signals for activation and predominantly induce differentiation of Foxp3⁺ regulatory T cells [20]. These phenomena are heavily influenced by components of the microbiota and their metabolites. However, certain components of the microbiota, such as filamentous bacteria, induce Th17 responses [21].

Once the Treg cells response is induced, such cells migrate to the lamina propria and suppress the action and MLN migration of the CD11c⁺CD103⁺CX3CR1⁺ DC; promoting tolerance [22].

Therefore, Tregs constitute a component of the immune system specialized in immune suppression; contributing to immune tolerance and homeostasis [23]. Tregs are CD4⁺T cells that commonly express the interleukin 2 receptor α -chain (CD25), although activated conventional T cells also transiently express it. The transcription factor known as forkhead box P3 (Foxp3) is required for the expression of multiple genes implicated in the development, maintenance, and function of most of the Tregs; thus it is considered their master regulator [24]. Tregs cells require T cell receptor (TCR) stimulation and co-stimulation for activation and can be divided in two subsets: natural Tregs (nTregs), derived from the Thymus, that emerge at early stages of development to suppress autoimmune responses and induced Tregs (iTregs), which are induced in the periphery following antigen exposure involving stimulation with the transforming growth factor β (TGF- β) and other phenomena [25]. It is known that malfunction or disruption of Tregs lead to autoimmune and inflammatory diseases in both humans and animals [26]. Therefore, an approach to treat such diseases is focused on expanding Treg populations able to restore homeostasis.

The following sections describe the plant-made vaccines developed thus far to induce tolerogenic responses, which lead to therapeutic effects in relevant diseases.

3. Advances in the development of plant-based tolerogenic vaccines

3.1 Inflammatory diseases

Allergies are the commonest chronic condition worldwide that are associated to Th2-mediated responses [27]. The development of allergy involves two phases: first, the immune system is sensitized with the allergen (priming phase); second, the immune system responds to an allergen challenge (effector phase) [28, 29]. Allergen immunotherapy has been proven to effectively limit the course of allergies. Immunotherapy typically consists in subcutaneous injections of ascending doses of crude allergen extracts over a period of 3-5 years in order to induce immune tolerance against the target allergen. The induced immune response is characterized by reduction of IgE antibodies and effector allergen-specific T cell responses [30-32]. Immunotherapies based on recombinant allergens are simpler, safer, and more efficacious treatments that might lead to the development of prophylactic vaccination approaches [30, 33]. Today, a number of research groups have developed immunotherapies based on transgenic plants expressing allergens.

A pioneering study reported by Krebitz et al. [34] consisted in the expression, in *Nicotiana benthamiana* via viral transfection, of the model allergen Bet v 1 (major birch pollen allergen) with yields up to 200 μ g/g of leaves. These authors demonstrated the antigenicity of the plant-

made Bet v 1 and proved that intraperitoneal immunization, in a murine model of type I allergy, induced IgE and IgG1 antibodies; a similar result to that obtained for the native protein. Thus the plant-made Bet v 1 is proposed as a low-cost immunotherapy against allergies [34].

Okada et al. [35] have described the expression, in rice seeds, of a chimeric protein comprising five T-cell epitopes of the Cry j 1 allergen. Plants expressing the target antigens were generated by *Agrobacterium*-mediated transformation, in which the transgene was under the control of the promoter and signal peptide of glutelin GluB-1, reaching expression levels up to 1.6 µg per mg of seed total proteins. The recombinant protein accumulated in protein bodies of rice endosperm as revealed by detection of GFP, which was fused to the recombinant allergen. In addition these authors presented evidence on the incapability of IgE antibodies, from allergic patients, to recognize the polypeptide Cry j 1; which is desirable in terms of safety since this suggests that the plant-made antigen will not exacerbate allergic responses [35].

In a study reported later, Takagi et al. [36] described the expression in the endosperm of rice of the polypeptide called 7Crp that includes seven T-cell epitopes of Cry j1 and Cry j2 allergens the Japanese cedar pollen. Transgenic lines were generated by *A. tumefaciens* transformation, the antigens were expressed under the control of the GluB-1 promoter and the KDEL signal peptide was fused to them to be retained in the endoplasmic reticulum. Expression levels reached up to 60 µg per grain. In order to evaluate the therapeutic relevance of 7Crp, mice were primed intranasally with the Cry j1 allergen before and after a 32-day period in which animals were fed with transgenic rice seeds containing the 7Crp protein. Although the plant-made therapy led to a 30% reduction in IgE levels with respect to the group treated with wild-type rice; this approach has the potential to effectively induce mucosal tolerance against pollen allergy, further studies on the clinical symptoms upon antigenic challenges are needed to determine its true efficacy [36].

The same research group reported a new version consisting of Crp-1 (Cry j I₂₇₇₋₂₉₀, identified as A1aB1b-Crp-1) and Crp-2 (Cry j II₂₄₆₋₂₅₉, identified as A1aB1b-Crp-2) fused to the soybean seed storage protein glycinin (A1aB1b). The antigens were expressed under the control of the GluB-1 promoter. The fusion proteins reached yields up to 0.5% of total seed protein. An immunization trial was performed in mice to determine the potential of these vaccine candidates. Mice were orally immunized with rice seeds containing 70 µg of A1aB1b-Crp-1 and A1aB1b-Crp-2 and subsequently challenged with total protein from cedar pollen. Mice treated with either vaccine candidate showed decreased IgE antibody responses and T CD4⁺

proliferative responses; as well as lower levels of Th2 cytokines (IL-4, IL-5, and IL-13). Moreover, the plant-made immunotherapy reduced clinical symptoms in an allergy mouse model [37].

Takagi et al. [38] reported a detailed characterization of the rice lines expressing 7Crp. Stable integration of the 7Crp gene was proven in T7 rice plants, which maintained the same protein productivity as compared with the T2 generation (based on Western blot analysis) with no nutrient content alterations. The *in vitro* digestibility studies revealed that 7Crp is completely digested after 15 min of incubation with pepsin and 60 min of incubation with pancreatin. 7Crp showed no N-glycans in its structure. Other studies have been focused on optimizing 7Crp accumulation by expressing it fused to Glutelin and using different endosperm-specific promoters [39]. When 7Crp was fused to the glutelin signal peptide and the KDEL ER signal retention, and expressed under the control of the promoter *GluB-1*, higher levels were achieved (60 µg/grain) when compared to the use of promoters from *GluB-4*, *Glb-1*, 10 kDa prolamin, and 16 kDa prolamin). Expression of the 7Crp-Glutelin fusion reached levels up to 80 µg/grain that were maintained up to T9 seeds. 7Crp accumulated in ER-derived protein bodies-I; likely mediated by the formation of disulfide bonds between the 7Crp peptide and cysteine-rich residues in prolamin storage proteins. In addition these authors observed increased levels of BiP and PDI chaperones in transgenic lines with notably higher accumulation of 7Crp in PB-I, which induces morphological changes in the protein body. No data on the immunomodulatory activity of these 7Crp versions has been reported, which will be a critical assessment to define their therapeutic potential.

The expression in rice of 3Crp or 3Crp-7Crp epitopes has also been assessed using two signal peptides, ER-derived PB-I or protein storage vacuole PB-II. PB-I-localized 3Crp-7Crp epitopes showed higher resistance after 120 min upon *in vitro* digestion with pepsin. An immunization scheme was performed to evaluate the induction of oral tolerance against the target allergen, which comprised daily administration of rice powder or the synthetic peptide for ten days. Mice were subsequently challenged with total proteins from the Japanese cedar pollen using aluminum hydroxide as adjuvant. Interestingly, the dose necessary to induce the abrogation of the allergy in mice fed with PBI-localized 3Crp-7Crp was 20-fold lower than that required for the case of the 3Crp synthetic peptide. Thus these authors hypothesized that bioencapsulation of 3Crp in PB-I provided by the rice endosperm represents an efficient delivery system allowing to decrease the therapeutic dose. Although the detailed mechanisms explaining the oral tolerance induced by this plant-based therapy remained to be characterized [40], the rice-

made 7Crp vaccine is promising and the current evidence justifies continuing with its preclinical evaluation.

In 2011, Lee and coworkers described the expression of the Der p 2 antigen from *Dermatophagoides pteronyssinus* 2, which is the major allergen that causes asthma. Tobacco plants were used as expression hosts and developed by *Agrobacterium*-mediated transformation following three different expression and accumulation strategies; observing the highest accumulation in the cytosol-targeting expression (0.5% TSP). The therapeutic effect of the plant-made Der p 2 was determined in BALB/c mice, they were sensitized by the i.p. route with 0.03 mg of rDer p2 using alum and pertussis toxin as adjuvants on days 0, 14 and 28; at the same time they were immunized on days 1 to 3 and 6 to 8 with plant extracts containing 0.1 mg of rDer p2, 0.1 mg of rDer p2 from yeast extract, or plant extract from the WT plant. Subsequently, mice were challenged on days 34 and 36 with 5 µg of rDer p2 by intratracheal injection. The mice group immunized with the plant-made rDer p2 showed decreased levels of IgE and IgG1 Der p2-specific antibodies; as well as lower levels of IL-4, IL-5, IL-13, and eotaxin when compared to the mice group immunized with the WT plant. Moreover, the plant-made rDer p2 inhibited the recruitment of eosinophils in airway and decreased airway inflammation and hyper-responsiveness. In addition, these authors reported increased population of CD4⁺ CD25⁺ Foxp3⁺ regulatory T cells in mediastinal and mesenteric lymph nodes from mice treated with the plant-made vaccine. Also, splenocytes showed decreased proliferation rates, as well as increased levels of IL-10, after stimulation with rDer p2. These therapeutic effects allow postulating that the Der p2 plant-based vaccine as a promising candidate in the treatment of allergic diseases having the expansion of Treg cells as one of the effector mechanisms. The perspectives for this candidate comprise safety assessment and determination of non-toxicological effects in preclinical trials [41].

The research group headed by Takaiwa reported the production of Derp1₄₅₋₁₄₅ (this sequence covers T cell dominant epitopes for human and mouse), fused to the signal peptide of glutelin and the ER retention signal KDEL, in rice seeds by *Agrobacterium*-mediated transformation. The N-glycosylation of this protein was confirmed by digestion with the Endo H enzyme. Der p 1 was deposited as aggregates in PB-I with yields up to 90 µg per grain. The prophylactic activity of the rice-made vaccine was assessed in mice first by administering orally the vaccine daily for seven days and a subsequent challenging with rDer p 1 seven and nine days after the last immunization. The authors observed an abrogation of allergenic responses in groups treated with the rice-made vaccine; this effect was likely mediated by inhibition of Th2 and allergen-specific IgE responses. No changes in Treg cells population were induced by the rice-

based vaccine suggesting that other mechanisms were involved in the oral tolerance induced; however, they remained to be elucidated [42].

Takaiwa et al. [30] reported the expression in transgenic rice seeds of T-cell epitopes from the Japanese cypress pollen allergens Chao 1 and 2 (6Chao) alone or in co-expression with 7Crp, 3Crp, or 7Crp/3Crp from the Japanese cedar. The antigens were fused to the ER retention signal KDEL under the control of different endosperm-specific promoters (GluB-1 or GluB-4). In addition, as an effort to generate a broad spectrum immunotherapy, 12Crp was expressed alone as secretory protein under the control of GluB-1 or GluB-4 promoters along with the respective signaling peptides. Rice transformation was mediated by *A. tumefaciens* and expression levels for individual peptides ranged from 0.6 to 2.75 mg/g mature seed. Moreover, the expression level of 3Crp peptides under three different promoters in separate expression cassettes increased when compared to using a single expression cassette. *In vitro* digestion with pepsin of 3Crp, 6Chao or 7Crp-3Crp-6Chao peptides in rice seeds required 30 min to achieve complete digestion, which is longer than the time required for glutelins digestion (15 min); suggesting that these peptides accumulated in ER-derived protein bodies. The recombinant antigens were mainly deposited as aggregates in ER-derived PB-I through interactions with prolamins rich in Cys residues by disulfide bonds. A critical prospect for these candidates of vaccines consists on determining their immunotherapeutic potential in pre-clinical evaluations in order to demonstrate their capability to induce broad immune responses.

Asthma is a chronic airway inflammatory disorder having many factors for its development such as genetic predisposition and environmental exposure to inhaled substances and particles. Although asthma is under-diagnosed and under-treated, it is estimated that 235 million of people currently suffer from asthma [43]; with children as the most affected group [44]. The conventional management of asthma controls the disease without achieving a cure. Immunotherapies are a viable strategy to alleviate this pathology, with tolerogenic vaccines against asthma under intensive research. Allergen-specific immunotherapy (SIT) is intended to induce clinical and immunological tolerance leading to a long-term clinical benefit and associated immunological parameters [45]; it is a safe, effective, and long-lasting therapy that modulates the immune process during the pathogenesis of the allergic disease with clinically safe, effective, and long-lasting outcomes. It is well known that enhanced Th2 immune responses and the production of cytokines such as IL-4, IL-13, and IL-5 contribute to the induction of allergy and asthma [46-48]; thus immunotherapies modifying the Th1/Th2 imbalance are considered promising for asthma.

In 2003 Smart et al. [49] reported evidence on the suppression of the onset of experimental asthma in mice by promoting oral tolerance through a tolerogenic vaccine candidate named SSA, which targets the sunflower seed albumin from *Helianthus annuus* L. SSA is a potent allergen that contains IgE-binding epitopes and possesses similar (34%) amino acid sequence to the known allergenic 2S albumin protein from the Brazil nut. SSA was expressed in transgenic seeds of *Lupinus angustifolius* L. Since some authors have stated that GM plants can induce allergy, to elucidate this point it was proven that the systemically consumption of SSA-lupin neither sensitize animals nor predispose to hypersensitivity. To induce experimental asthma, mice were i.p. sensitized with SSA and alum with a subsequent i.t. challenge using either SSA or the vehicle control. Purified SSA induces experimental asthma and promotes an inflammatory response comparable to that of the established disease model Ag OVA. Mice were orally administered with lupin or SSA-lupin meal and subsequently primed i.p. and i.t. challenged with SSA to determine the type of immune response induced against SSA. The results indicated that an attenuation of mucus hypersecretion, pulmonary eosinophilic inflammation, and airways hyperreactivity (AHR) were achieved in groups treated with the plant-made SSA. The suppression of experimental asthma correlated with the expansion of CD4⁺ CD45^{low} suppressor T cell population and IFN- γ production. This was the first study demonstrating that plant-based tolerogenic vaccines promote suppressive immune responses able to attenuate experimental asthma.

The house dust mites *Dermatophagoides pteronyssinus* (Der p) cause chronic allergic diseases such as bronchial asthma, rhinitis, and atopic dermatitis. The allergen Der p 1, produced in transgenic rice seeds, consisted of the Der p residues 45-145 comprising the major mouse and human T cell epitopes of this protein. The amount of Der p 1 expressed was about 4.5 μ g/mg of seeds. Mice were fed with approximately 50 mg Der p 1 over a period of 7 days (6.8 mg per day). The oral administration to mice prior to a systemic challenge with Der p 1 induced oral tolerance associated with an inhibition of bronchial hyper-responsiveness (BHR), inhibition of allergen-specific T helper 2 (Th2) cytokine synthesis (IL-4, IL-5, and IL-13), and a decrease of allergen-specific IgE responses. When mice were secondary immunized with unrelated antigens, the levels of IgE and IgG were oral-specific to Der p1 [50].

Li et al. [51] used *Nicotiana benthamiana* plants to transiently express tR8, a His-tagged protein derived from the major allergen group 1 of house dust mites (*Dermatophagoides farinae* and *D. pteronyssinus* species). Purified tR8 was obtained at concentrations of 4.54 $\pm$ 0.142 mg/g 7 days post-transformation. The plant-made tR8 retained the capacity to bind IgE in an ELISA assay using sera from *D. farinea*-allergic patients. BALB/c mice were

intraperitoneally injected with three doses of 10 µg of relevant allergen and 2% Al(OH)₃ as adjuvant. After the third dose, mice were challenged by nebulized inhalation of an allergen suspension (0.5 µg/mL) for 30 min during 7 successive days. Mice were subsequently subjected daily to allergen-specific immunotherapy by i.p. injection of rProDer f 1, tProDer f 1, and tR8 (100 µg/mL). The effects of tR8 in a mice model of asthma comprised a predominant Th1 response since IFN-γ and IL-2 were secreted at high levels whereas Th2 cytokines were greatly reduced; suggesting that tR8 enabled activation and expansion of Th1- and Treg cells. IgG1 levels were also significantly down-regulated in the tR8 group but IgG2a levels were strikingly raised when compared to other experimental groups. These findings suggest that tR8 can competently rectify the Th1/Th2 imbalance [51].

3.2 Autoimmune diseases

Insulin-dependent diabetes or juvenile-onset diabetes, currently known as Type 1 diabetes (T1D), affects 20–40 million persons worldwide according to the International Diabetes Federation [52]. Estimations about the future prevalence indicate that over 552 million of persons will be affected by this disease by 2030. T1D is about 5 to 10% of all diabetes cases [53]. A cellular-mediated autoimmune destruction of the beta-cells of the pancreas is a key physiopathological event associated to T1D development. The incidence of T1D has been explained by genetic and environmental factors. Among the environmental factors associated to T1D onset are infections with viral pathogens, diet, obesity, and insulin resistance; among others [54-56]. Since T1D is considered an autoimmune disease, understanding immune system alterations that lead to pancreatic inflammation and beta cell destruction is critical to develop therapies.

Although a detailed mechanistic view on the diabetes physiopathology is not available thus far, several studies have demonstrated that the letdown of β cells is mediated by the expansion of β cell-reactive T cells and the generation of a pro-inflammatory microenvironment. The presence of autoantibodies against islet antigens, such as human Glutamic acid decarboxylase (GAD65), protein tyrosine phosphatase IA2 (IA-2AA), and Znt8; has also been associated to disease onset [57].

It is known that destruction of β cells is triggered via the innate immunity cells such as the immature macrophages or the dendritic cells (DCs) that in the steady-state express pattern-recognition receptors (PRRs) that recognize pathogen-associated molecular patterns (PAMPs) from pathogens or danger-associated molecular pattern molecules (DAMPs). Interestingly, the expression in animals of different types of PRRs such as TLR7, TLR9, MyD88, and NLPR3

predispose to T1D [58-62]. The recognition of PRRs leads to the activation of transcription factors (e.g. NF- κ B and IFN-regulatory factors) and secretion of inflammatory cytokines. The inflammatory environment stimulates innate function having as consequence an increase in IL-1 β and TNF- α levels [63]. All these conditions constitute a stress for the islet beta cell and favor activation of naïve T cells, which attracts more phagocytic cells and effector T cells within the pancreatic islets to directly destroy the β cells [64].

Although several advances on diabetes treatment have been achieved, no cure is available and patients depend on insulin therapy and the control of metabolic complications that include retinopathy, nephropathy, neuropathy, and cardiovascular disease [65]. Regarding the efforts to develop T1D therapies targeting autoimmune pathogenesis, several studies on plant-based approaches have been conducted. Transgenic potato plants were generated to express human insulin fused to CTB via *A. tumefaciens*-mediated transformation with yields up to 0.1% TSP [66]. In oral administration schemes the plant-made insulin chimera induced oral, Th2 mediated tolerance in non-obese diabetic (NOD) mice treated with a 20 μ g dose of plant-made insulin once a week for 5 weeks. However, the vaccine did not reduce insulinitis and showed no significant diabetes suppression in the long term.

Another autoantigen associated to diabetes, GAD65, has been expressed in tobacco and carrot using *Agrobacterium*-mediated transformation [67]. The plant-made GAD65 preserved conformational autoantibody epitopes, which suggested a correct protein folding. Yields for transgenic tobacco and carrot were up to 0.01% and 0.04% TSP, respectively. A chimeric protein named GAD67/GAD65 was generated by replacing the NH₂-terminal region of hGAD65 by a homologous region of rat GAD67 [68]. Human GAD65 (hGAD65) was also targeted. Tobacco plants expressing the target antigens were generated by the *Agrobacterium*-based methodology and recombinant proteins were accumulated in the cytosol. Transient expression was assessed in parallel using *N. benthamiana* plants and a Potato virus X vector (pPVX). Stably transformed tobacco plants accumulated the GAD67/65 antigen at 0.19 % TSP, whereas hGAD65 reached yields of 0.04% TSP. Transient transformation allowed yields of up to 2.2% of TSP. A mutated version of GAD65 (GAD65mut) was also expressed in tobacco in which the lysine residue responsible for binding the cofactor pyridoxal 5'-phosphate (PLP) was replaced by arginine [69]. This mutation allowed obtaining a catalytically inactive autoantigen that retained immunogenicity and binding capacity to GAD65 autoantibodies in serum from patients with T1D. Both GAD65mut and GAD67/65mut accumulated at higher levels (0.26% TSP) when compared to the non-mutant proteins, with no benefits in yields when accumulated in the ER. No functional studies in mice have been reported for these vaccines targeting GAD.

In 2006, Li et al. expressed the β -chain of human insulin as a CTB fusion called CTB–InsB3 in an arrangement including three tandem repeats of the β -chain [70]. Transgenic tobacco plants were generated using a cultivar with no nicotine and low levels of alkaloids. Yields of CTB–InsB3 were in the 0.08-0.11% TSP range. The plant-made CTB–InsB3 retained the ability to bind GM1 suggesting a pentameric structure. No animal assays were conducted.

Another approach explored in this field consisted in the individual expression of IL-4 and GAD65 in transgenic tobacco plants [71]. *In vitro* studies revealed that the plant-made IL-4 is functional since it induced mast cell proliferation. The authors used 4-week old NOD mice to determine if the treatment with plant-made biopharmaceuticals inhibits insulinitis before diabetes establishment. Test animals were fed during 5 weeks with biomass from plants expressing IL-4 or GAD65; or a combination of both materials. Insulinitis was decreased in the groups treated with IL-4 plus GAD65 (mean values of 30% for control group fed with placebo vs. 8% for the experimental treatment). Treatments with the individual biopharmaceuticals induced no significant changes in insulinitis scores.

In another effort, GAD65mut and its derivatives lacking the first 38, 69, and 87 codons were expressed using the viral vectors pK7WG2 and MagnICON; as well as the conventional stable expression at the nuclear level [72]. The MagnICON system was superior in yields, with levels up to 226.9 $\mu\text{g/g}$ of fresh leaves weight. Furthermore, $\Delta 87\text{GAD65mut}$ was expressed using the MagnICON system and its expression showed the greatest solubility compared to GAD65mut, 7-fold less turbidity, 32% less phenolic compounds, and 30% less host proteins in the extract in a detergent-free extraction buffer.

Rheumatoid arthritis (RA) is a chronic autoimmune disease that causes the gradual destruction and loss of joints function; besides vascular, metabolic, bone, and psychological affections. RA occurs at any age; with a greater number of cases in women than men that affects 1% of the world population [73]. Although the physiopathology is still not completely clear, autoimmune responses are well described; mainly mediated by innate immunity that includes activation of complement mediated by immune complexes, as well as adaptive immunity such as the induction of antibodies against modified post-translationally self-antigens and dysregulated cytokine networks [86]. In addition, the disease can be enhanced by the activation of chondrocytes and osteoclasts [74, 75]. Treatments for RA have been focused on targeting some of the mediators of the autoimmune responses. For instance, inhibitors of TNF- α and IL-6 have been used with notable results. Recently, the JAK/STAT signaling pathway has been pointed out as a relevant mechanism that favors RA progression; converting the Janus kinase

inhibitors into promising targets, which have been tested in clinical trials [76]. The development of antigen specific therapies would be ideal since fewer side effects would be expected due to therapy specificity. Among the specific autoantigens associated to RA, it is well documented that RA patients possess T cells that are reactive to Type II collagen (CII); in particular to the epitope comprising amino acids 256-271. Interestingly, down-regulation of such autoreactive T cells has been achieved through the use of variants of that epitope carrying amino acid substitutions (altered peptide ligands, APL) [77].

The race for developing RA treatments requires with no doubts an expansion of the therapeutic alternatives and plant-based vaccines candidates have been postulated. Rice plants accumulating CII256-271, and its analog peptide APL6, were developed by Lizuka et al. [78]. The antigens were expressed and accumulated in protein storage vacuoles as fusion proteins comprising the major rice storage protein glutelin and three tandem repeats of either CII256-271 or APL6; achieving yields ranging 7-24 mg/g seeds. The rice-based vaccines were orally administered to a mice RA model to evaluate therapeutic effects. RA was induced in mice by administering bovine type II collagen along with Freund's complete adjuvant. Mice groups were subsequently treated once a day for 2 weeks to determine the therapeutic effects of the vaccines. Importantly, the clinical scores of mice treated with the APL6 rice-based vaccine were significantly reduced and the appearance of the disease was delayed in contrast to those treated with the rice-based CII256-271 vaccine or standard rice. The authors presented data that correlated the production of IL-10 from CII-specific CD4+CD25- T cells in cultures of spleen and inguinal lymph nodes (iLN) cells. Therefore, the rice-based vaccine is considered a promising candidate for the development of innovative therapies against RA.

In a subsequent study, the same research group explored new candidates based on the APL4, APL6, APL7, and CII256-271 epitopes following the same expression approach described above; achieving yields up to 0.93, 3.69, 1.35, and 1.08 mg/g seed; respectively. Functional evaluation of the vaccine candidates was focused on determining their prophylactic effects against a CII challenge. For this purpose, mice groups were orally administered once a day for 14 days with the vaccine candidates or standard rice material; followed by an intradermal challenge with the CII antigen. Prophylactic effects were only observed in mice fed with the APL7-based vaccine, which also showed increased levels of IL-10-producing cells in spleen and iLN; while no changes in IFN- γ , IL-17, and IL-2 production were observed. These findings suggest that IL-10 produced by CD4 + CD25- T cells is mechanistically involved in the observed prophylactic effects [79].

The previously patented fusion protein CTA1(R7K)-COL-DD, expressed in bacteria, has shown remarkable results in conferring protection to mice with collagen-induced arthritis. A study was carried out to investigate if the tolerogenic protein could be expressed in edible plants to allow for their oral use. The CTA1(R7K)-COL-DD transgene was assembled in the pGreen vector containing the CaMV 35S promoter and the CaMV polyA terminator; *Arabidopsis thaliana* plants were transformed by floral immersion in *Agrobacterium*. The protein analysis showed yield of 30 ng/mg of biomass or 25 ng/ μ g of TSP. Male DBA/1 mice with collagen-induced arthritis were fed with transgenic or wild-type *A. thaliana* plants ad libitum once every week for 4 weeks. The treated mice reduced the clinical scores significantly, the biomarkers of inflammation, and anti-collagen IgG responses; the counting of effector T cells CD4⁺ in the spleen and lymph nodes was also reduced. Finally, a reduction in IFN γ , IL-13, and IL-17A production was recorded with an increase in the production of IL-10; suggesting a reduced Th1, Th2, and Th17 activity and elevation of regulated functions of IL-10 and regulatory T cells (Treg) [80].

Another proposed target for the search of immunotherapies against RA is an epitope of the enzyme glucose-6-phosphate isomerase (GPI325-339) or its analogue APL12. Target epitopes were fused in a three tandem repeat arrangement to glutelin and expressed in rice endosperm by using specific seed promoters. The accumulation of proteins reached 3.3 mg/g of seeds for hGPI325-339 and 3.8 mg/g for APL12 as glutelin fusion proteins. Transgenic rice lines were administered orally to DBA/1 mice (400 μ g once a day for 2 weeks); at the end of the prophylactic treatment, mice were challenged with the GPI antigen to induce arthritis. The effect of the prophylactic treatment of GIA mice with transgenic APL12 rice seeds was observed in the significant reduction in severity of arthritis and anti-GPI serum antibody titers; in addition, histopathological arthritis scores improved and IL-17 production decreased when compared to mice treated with nontransgenic rice. GIA mice treated with APL12 rice showed increased expression of Foxp3 and GITR in CD4⁺ CD25⁺ Foxp3⁺ cells in the spleen when compared to mice not treated with transgenic rice [81].

3.3 Allo-response to therapeutic proteins

Hemophilia is a hemorrhagic disorder caused by mutations in the genes encoding for the coagulation factors VIII (hemophilia A) and IX (hemophilia B) [82]. Hemophilia A affects 1 in 5000 male live births, whereas hemophilia B has a frequency of 1 in 30,000. Factors VIII and IX are part of the intrinsic blood coagulation pathway and patients may be mildly (defined by

factor plasma levels from 6 to 40%), moderately (2 to 5%), and severely (1% or less) affected [83]. Although the coagulation mechanisms were classified in two separate pathways, there is evidence on the role of both factors as precursors in thrombin generation. After an injury, the generation of factor Xa is preceded by the activation of the tissue factor complex and factor VIII. However this production must be enhanced by factors IX and VIII to allow completing the coagulation process. Therefore the lack of factors VIII and IX leads to continued bleeding since there is no sufficient factor Xa to sustain hemostasis [84]. Hemophilia is currently treated by intravenous infusion of recombinant or plasma-derived coagulation factor VIII (F.VIII) or factor IX (F.IX). Nevertheless, in many patients, humoral responses against F.VIII and F.IX are developed; representing a serious situation. Some of the potential strategies that have been explored to address this problem comprise the induction of tolerance through oral delivery of coagulation factors [85].

Verma and colleagues [86] applied transplastomic technologies to develop a potential oral tolerogenic therapy based on the delivery of F.IX by tobacco cells. Tobacco plants were transformed by biolistics to target the expression of F.IX in chloroplasts; leading to the accumulation of high levels of human F.IX in the form of a fusion protein having the cholera toxin B subunit (CTB) as carrier. Two versions of fusion proteins were tested having glycine-proline-glycine-proline hinge to prevent steric hindrance (CTB-FIX) or a furin cleavage site plus GPGP hinge (CTB-FFIX), they accumulated at levels up to 0.19 and 3.8% TSP (0.4 mg/g of leaf tissue); respectively. The correct assembly of the pentameric CTB-based chimeras was confirmed by a GM1-ELISA assay for both versions. These authors used the mice model C3H/HeJ $F9^{-/-}$ in which the deletion of the F9 gene on the genetic background of C3H/HeJ mice was performed and serves as a model of hemophilia B capable of developing robust immune responses to human FIX (hF.IX) therapy. Three experimental groups received orally twice a week for 2 months (250 mg / dose) CTB-FIX, CTB-FFIX transplastomic plant, or non-transformed (WT) leaves. One month later, a weekly dose (1 IU per mouse) of hF.IX was administered intravenously for 2 months. Two additional groups not treated with plant tissues received intravenous injections of hF.IX alone. At the end of the study mice fed with CTB-FIX- and CTB-FFIX transplastomic lines survived the initial 2-month period of eight weekly hF.IX injections and even subsequent injections, without allergic signs or anaphylactic reactions; with survival rates of 90 and 93%, respectively. Test mice were protected by suppression of the neutralizing IgE and IgG1 titers.

To dissect the immune mechanism behind the tolerance induced to F.IX, in another study; plant cells expressing CTB-FFIX (125 mg of frozen transgenic plant cells containing 5 µg of CTB-

FFIX) were administered to C3H/HeJ $F9^{-/-}$ mice in a scheme of two doses per week over a 2-month period. Mice were subsequently challenged one month post-tolerization with 1 IU hF.IX IV once a week for 2 months. Evidence on the capture of the antigen by dendritic cells at the level of the small intestine and by F4/80⁺ cells in the duodenum was generated. In regard to the immunomodulatory responses induced by the plant-made CTB-FFIX, an increase in the following parameters was observed: number of CD103⁺ tolerogenic and plasmacytoid dendritic cells, cell polarization of CD4⁺CD25⁻LAP⁺, expression of IL-10 and transforming growth factor- β (TGF- β), and T cells regulating CD4⁺CD25⁺ anti-FIX responses [87].

Looking to develop an attractive platform for the direct formulation of oral vaccines; the use of lettuce, which unlike tobacco lacks of toxic metabolites for humans, as expression host and delivery vehicle of CTB-FFIX was assessed. Transplastomic lettuce lines were developed by biolistics. The lettuce-made CTB-FFIX was able to induce tolerance in mice fed with CTB-FFIX, an evidence in the antigen delivery to the intestinal immune system was generated; as well as the induction of regulatory T cells LAP⁺. Moreover the production of inhibitors/IgE and anaphylactic reactions against hF.IX were prevented in mice treated with the lettuce-made CTB-FFIX [88]. Interestingly, a further evaluation of the lettuce-based vaccine consisted in its administration to dogs carrying a mutation in the F9 gene (UNC-Chapel Hill strain; Herzog and colleagues [89]). Test animals (n=4) were treated orally twice a week for 13 weeks with food mixed with lyophilized lettuce cells (0.3 mg/kg dose of the CTB-FIX antigen). Three of the treated dogs presented robust suppression of inhibitor IgG and IgE upon an intravenous hF.IX treatment with no side effects. This successful trial in a big animal model opens a new avenue to advance in the justification for projecting clinical trials.

Another approach in this field consisted in the fusion to CTB of the F.VIII heavy chain or the C2 domain, rendering the CTB-HC and CTB-C2 chimeras; respectively. This design is convenient since the full length F.VIII is a large size protein. The genes coding these candidates were inserted into the chloroplast tobacco cells by biolistics and the generated lines reached yields up to 80 and 370 $\mu\text{g/g}$ of fresh leaves. CTB-HC and CTB-C2 chimeras were folded correctly and 125 mg of leaves containing $\sim 5 \mu\text{g}$ HC and $6 \mu\text{g}$ C2 per dose/mouse were administered orally to F8e16^{-/-} mice on a C57BL6/129 genetic background. Animals were treated with two doses per week for 2 months. During the second month a concentrate of F.VIII was administered intravenously once a week (1 IU/mouse). Interestingly, mice fed with WT plant tissues formed F.VIII inhibitors at very high titers, whereas mice fed the transgenic mixture suppressed the formation of anti-F.VIII IgG1, IgG2a, and IgG2b. The analysis of splenocytes by flow cytometry showed a positive regulation of immunosuppressive cytokines TGF- β and IL-10; as well as an

active suppression mechanism associated with the induction of CD4⁺ CD25⁺ and CD4⁺ CD25⁻ T cells [90].

4. Expert opinion

In view of the demand of new therapies to fight immune-related diseases, new platforms for the production and delivery of tolerogenic antigens are of key importance for medical biotechnology.

There is a diverse group of therapies under clinical and preclinical development to fight against autoimmune and inflammatory diseases. For instance, therapeutic antibodies produced in cell lines are currently used in the clinic [91]; as well as Intravenous immunoglobulin (IVIG) obtained from healthy donors [92]. Moreover, many new candidates are under preclinical evaluation. For instance, new schemes based on antibodies targeting IL-2 [93]; as well as epitope-based immunotherapies are promising avenues that can lead to new treatments in the clinic [94-96].

In this context, plant-based platforms have been adopted for this purpose; auguring a path for the generation of new products that will become a new alternative in the clinic. The analysis made in the present review indicates that plant-made vaccines offer a promising approach to down-modulate immune responses associated to several pathologies, making viable the development of attractive tolerogenic vaccines (Table 1). The majority of the candidates have been assessed in test animals thus far, providing valuable data on their efficacy. Despite this progress, the following goals will be critical to advance on the road of developing new therapies: (i) performing toxicological and stability analyses, which are required to conclude preclinical evaluation of the promising candidates and justify their evaluation in clinical trials; (ii) establishing the scale-up approach to produce sufficient plant material to cover the test schemes in humans; in this regard systems to produce plant-made antigens under GMPs have been described [97], (ii) attracting investment by governments or Biopharma companies.

There is solid evidence on the fact that biopharmaceuticals encapsulated in plant cells are stable products that avoid the cold-chain for storage, decrease costs, and mediate the oral delivery of the active drug; which decrease logistics costs and offers friendly and simple administration [98, 99]. However, optimizing antigens expression in attractive plant species is required since they have been expressed in tobacco and *N. benthamiana*, which are not appropriate hosts for the formulation of purification-free oral vaccines. Therefore, moving

these vaccination models to edible crop species with proven potential to serve as vaccines biofactories, such as lettuce and carrot, is a key goal for the field. The advances in expression approaches in plants offer a realistic approach for vaccine formulation, mainly by the use of efficient promoters and seed accumulation strategies. Viral vector-based systems offer rapid and consistent yields [100, 101]; an ideal situation for the initial phases of vaccine development [102, 103].

Although several tolerogenic therapies based on conventional technologies are under development, the plant-based platforms offer several advantages in contrast to mammalian and bacterial cell production: they do not replicate human pathogens and they are produced at very low costs [3, 104, 105]. These advantages contrast with the case of bacterial systems (namely *E. coli*), which contains endotoxins and thus exhaustive purification of the product must be performed to obtain appropriate injectable products. Other limitation of bacterial expression is the limitation in terms of biosynthetic capacity since bacteria do not perform complex post-translational modifications, such as glycosylation, that are often required to render functional biopharmaceuticals. In the case of mammalian expression systems and biologicals obtained from donors, the risk of contamination with pathogens (e.g. prions and viruses) is a prominent drawback [106]. Mammalian cell lines also imply slow growth and fragility; with relatively high production time and cost [107]. Insect cell lines have also been successfully applied to biopharmaceutical production with the advantage, over bacterial systems, that post-translational modifications occur. In contrast to mammalian cell lines, insect cells are less demanding and grow to higher densities. Nonetheless insect cells provoke significant variations in the glycosylation patterns and the culture medium is expensive, which increases the biopharmaceutical production cost [108].

Although the use of a plant-based vaccines in humans has not been approved so far, several prototypes with high potential have been developed. In parallel, the approval of one plant-made biopharmaceutical for human use has been achieved: the case of glucocerebrosidase, an enzyme used as a replacement in Gaucher's disease; which is produced in carrot cells as an efficient glycoform that does not require expensive in vitro treatment as the homologue produced in mammalian cells [109]. Interestingly, current research is focused in studying the oral delivery of glucocerebrosidase in a clinical phase II trial (www.protalix.com). The results showed that patients with Gaucher's disease have glucocerebrosidase levels similar to healthy control subjects when consuming a juice containing carrot cells with glucocerebrosidase [110]. Another highlighted achievement in this field includes the case of ZMapp™, a monoclonal antibodies cocktail to treat Ebola produced in *N. benthamiana*; which was emergently used to

treat 7 patients during the 2014 Ebola outbreak based on its efficacy in rhesus macaques [111]. A subsequent clinical trial was conducted in the U.S., Liberia, Sierra Leone, and Guinea for ZMapp™ to assess its safety and efficacy. Moreover, other antibodies against infectious agents (e.g. anthrax, Respiratory syncytial virus, West Nile virus, and Human immunodeficiency virus) and Non-Hodgkin's Lymphoma are under evaluation. This relevant topic is reviewed elsewhere [112].

In conclusion recent achievements on the field of plant-made biopharmaceuticals in combination with the evidence on the generation of functional tolerogenic therapies, assessed in preclinical trials, augur the emergence of new affordable therapies to treat autoimmune diseases and allergies in the near future.

Funding

Current investigations from the group are supported by CONACYT/México (grant CB-256063 to SRM).

Declaration of Interests

S Rosales-Mendoza was supported by CONACYT/Mexico (grant CB-256063 to SRM). The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

Reviewer Disclosures

Peer reviewers on this manuscript have no relevant financial relationships or otherwise to disclose.

REFERENCES

***of interest**

****of considerable interest**

[1] Kesik-Brodacka M. Progress in biopharmaceutical development. *Biotechnol Appl Biochem*. 2018;65(3):306-322.

[2] Hefferon K. Plant-derived pharmaceuticals for the developing world. *Biotechnol J*. 2013;(10):1193-202.

[3] Govea-Alonso DO, Cardineau GA. Principles of Plant-Based Vaccines. In: Rosales-Mendoza S, editor. *Genetically Engineered Plants as a Source of Vaccines Against Wide Spread Diseases, An Integrated View*. New York (NY): Springer; 2014. p. 1-14.

***Contains a good introduction on the plant-based vaccines topic**

[4] Curtiss R III, Cardineau GA. Oral immunization by transgenic plants. 1997 United States Patent 5,654,184.

[5] Gleba Y, Marillonnet S, Klimyuk V. Engineering viral expression vectors for plants: the 'full virus' and the 'deconstructed virus' strategies. *Curr Opin Plant Biol*. 2004;7:182-8.

[6] Oey M, Lohse M, Kreikemeyer B, et al. Exhaustion of the chloroplast protein synthesis capacity by massive expression of a highly stable protein antibiotic. *Plant J*. 2009;57:436-45.

[7] Su J, Zhu L, Sherman A, Wang X, Lin S, Kamesh A, Norikane JH, Streatfield SJ, Herzog RW, Daniell H. Low cost industrial production of coagulation factor IX bioencapsulated in lettuce cells for oral tolerance induction in hemophilia B. *Biomaterials*. 2015;70:84-93.

****Represents a good example on the production of tolerogenic agents in plants**

[8] Pillet S, Aubin É, Trépanier S, et al. A plant-derived quadrivalent virus like particle influenza vaccine induces cross-reactive antibody and T cell response in healthy adults. *Clin Immunol*. 2016;168:72-87.

[9] Lomonosoff GP, D'Aoust MA. Plant-produced biopharmaceuticals: A case of technical developments driving clinical deployment. *Science*. 2016;353(6305):1237-40.

[10] Landry N, Ward BJ, Trépanier S, et al. Preclinical and clinical development of plant-made virus-like particle vaccine against avian H5N1 influenza. *PLoS One*. 2010;5(12):e15559.

[11] Tekoah Y, Shulman A, Kizhner T, et al. Large-scale production of pharmaceutical proteins in plant cell culture-the Protalix experience. *Plant Biotechnol J*. 2015;13(8):1199-208.

***A good example on the industrial adoption of plant-made biopharmaceuticals**

[12] PREVAIL II Writing Group.; Multi-National PREVAIL II Study Team., Davey RT Jr, Dodd L, Proschan MA, Neaton J, Neuhaus Nordwall J, Koopmeiners JS, Beigel J, Tierney J, Lane HC, Fauci AS, Massaquoi MBF, Sahr F, Malvy D. A Randomized, Controlled Trial of ZMapp for Ebola Virus Infection. *N Engl J Med*. 2016;375(15):1448-1456.

[13] Wambre E, Jeong D. Oral Tolerance Development and Maintenance. *Immunol Allergy Clin North Am*. 2018;38(1):27-37.

[14] Hamad A, Burks W. Oral tolerance and allergy. *Semin Immunol*. 2017;30, 28-35.

- [15] Mowat AM. Anatomical basis of tolerance and immunity to intestinal antigens. *Nat Rev Immunol*. 2003;3(4):331-41.
- [16] Jutel M, Kosowska A, Smolinska S. Allergen Immunotherapy: Past, Present, and Future. *Allergy Asthma Immunol Res*. 2016;8(3):191-7.
- [17] McDole JR, Wheeler LW, McDonald KG, et al. Goblet cells deliver luminal antigen to CD103+ dendritic cells in the small intestine. *Nature*. 2012;483(7389):345-9.
- [18] Berin MC, Shreffler WG. Mechanisms Underlying Induction of Tolerance to Foods. *Immunol Allergy Clin North Am*. 2016;36(1): 87-102.
- [19] Schiavi E, Smolinska S, O'Mahony L. Intestinal dendritic cells. *Curr Opin Gastroenterol*. 2015; 31(2):98-103.
- [20] Aliberti J. Immunity and tolerance induced by intestinal mucosal dendritic cells. *Mediators Inflamm*. 2016;2016:3104727.
- [21] Mann ER, Landy JD, Bernardo D, et al. Intestinal dendritic cells: their role in intestinal inflammation, manipulation by the gut microbiota and differences between mice and men. *Immunol Lett*. 2013;150:30-40.
- [22] Hadis U, Wahl B, Schulz O, et al. Intestinal tolerance requires gut homing and expansion of FoxP3+ regulatory T cells in the lamina propria. *Immunity*. 2011;34(2):237-46.
- [23] Sakaguchi S, Wing K, Miyara M. Regulatory T cells - a brief history and perspective. *Eur J Immunol* 2007;37:S116-23.
- [24] Dominguez-Villar M1, Hafler DA2,3. Regulatory T cells in autoimmune disease. *Nat Immunol*. 2018;19(7):665-673.
- [25] Singer BD, King LS, D'Alessio FR. Regulatory T cells as immunotherapy. *Front Immunol* 2014;5:46.

****Highlights the relevance of Treg cells in immunotherapy**

- [26] Zhao H, Liao X, Kang Y. Tregs: Where We Are and What Comes Next? *Front Immunol*. 2017;8:1578.
- [27] American Academy of Allergy, Asthma & Immunology [Internet]. Milwaukee, WI: AAAAI; 2017 [cited 2018 Oct 04]. Descriptive note for allergies, Available from: <http://www.aaaai.org/conditions-and-treatments/allergies>
- [28] Sampson HA, Aceves S, Bock SA, et al. Food allergy: a practice parameter update-2014. *J Allergy Clin Immunol* 2014;134(5):1016-25.e43.
- [29] Rael E. Allergen Immunotherapy. *Prim Care*. 2016;43(3):487-94.
- [30] Takaiwa F, Yang L. Development of a rice-based peptide vaccine for Japanese cedar and cypress pollen allergies. *Transgenic Res*. 2014;23(4):573-84.
- [31] Frew AJ. Allergen immunotherapy. *J Allergy Clin Immunol*. 2010;125(2 Suppl 2):S306-13.

[32] Larche M, Akdis CA, Valenta R. Immunological mechanisms of allergen-specific immunotherapy. *Nat Rev Immunol*. 2006;6:761–771.

[33] Ma S, Liao YC, Jevnikar AM. Induction of Oral Tolerance with Transgenic Plants Expressing Antigens for Prevention/Treatment of Autoimmune, Allergic and Inflammatory Diseases. *Curr Pharm Biotechnol*. 2015;16(11):1002-11.

[34] Krebitz, M, Wiedermann U, Essl D, et al. Rapid production of the major birch pollen allergen Bet v 1 in *Nicotiana benthamiana* plants and its immunological in vitro and in vivo characterization. *FASEB J*. 2000;14(10):1279-88.

[35] Okada A, Okada T, Ide T, et al. Accumulation of Japanese cedar pollen allergen, Cry j 1, in the protein body I of transgenic rice seeds using the promoter and signal sequence of glutelin GluB-1 gene. *Mol Breed*. 2003;12:61-70.

[36] Takagi H, Saito S, Yang L, et al. Oral immunotherapy against a pollen allergy using a seed-based peptide vaccine. *Plant Biotechnol J*. 2005;3:521–533.

[37] Takagi H, Hiroi T, Yang L, et al. A rice-based edible vaccine expressing multiple T cell epitopes induces oral tolerance for inhibition of Th2-mediated IgE responses. *Proc Natl Acad Sci USA*. 2005;102:17525-30.

****An example of a vaccine targeting allergies, evaluated at the preclinical level**

[38] Takagi H, Hirose S, Yasuda H, et al. Biochemical Safety Evaluation of Transgenic Rice Seeds Expressing T Cell Epitopes of Japanese Cedar Pollen Allergens. *J Agric Food Chem*. 2006; 54:9901-9905.

[39] Takaiwa F, Hirose S, Takagi H, et al. Deposition of a recombinant peptide in ER-derived protein bodies by retention with cysteine-rich prolamins in transgenic rice seed. *Planta* 2009;229:1147–1158.

[40] Takagi H, Hiroi T, Hirose S, et al. Rice seed ER-derived protein body as an efficient delivery vehicle for oral tolerogenic peptides. *Peptides* 2010;31:1421-1425.

[41] Lee CC, Ho H, Lee KT, et al. Construction of a Der p2-transgenic plant for the alleviation of airway inflammation. *Cell Mol Immunol*. 2011;8:404-414.

[42] Suzuki K, Kaminuma O, Yang L, et al. Prevention of allergic asthma by vaccination with transgenic rice seed expressing mite allergen: induction of allergen-specific oral tolerance without bystander suppression. *Plant Biotechnol J*. 2011;9(9):982-90.

****An example of a vaccine targeting allergies, evaluated at the preclinical level**

[43] World Health Organization [Internet]. Geneva, Switzerland: WHO; 2017 [cited 2018 Oct 14] Asthma Fact sheet N° 307, Available from: <http://www.who.int/mediacentre/factsheets/fs307/en/>

[44] Bisgaard H, Szefer S. Prevalence of asthma-like symptoms in young children. *Pediatr Pulmonol*. 2007;42(8):723-8.

[45] Yukselen A, Kendirli SG. Role of immunotherapy in the treatment of allergic asthma. *World J Clin Cases*. 2014;2(12):859-65.

[46] Deo SS, Mistry KJ, Kakade AM, et al. Role played by Th2 type cytokines in IgE mediated allergy and asthma. *Lung India*. 2010;27(2):66-71.

[47] Mishan-Eisenberg G, Borovsky Z, Weber MC, et al. Differential regulation of Th1/Th2 cytokine responses by placental protein 14. *J Immunol*. 2004;173(9):5524-30.

[48] Ngoc PL, Gold DR, Tzianabos AO, et al. Cytokines, allergy, and asthma. *Curr Opin Allergy Clin Immunol*. 2005;5(2):161-6.

[49] Smart V, Foster PS, Rothenberg ME, et al. A plant-based allergy vaccine suppresses experimental asthma via an IFN-gamma and CD4+CD45RB^{low} T cell-dependent mechanism. *J Immunol*. 2003;171(4):2116-26.

****An example of a plant-based vaccine targeting asthma, evaluated at the preclinical level**

[50] Suzuki K, Kaminuma O, Yang L, et al. Prevention of allergic asthma by vaccination with transgenic rice seed expressing mite allergen: induction of allergen-specific oral tolerance without bystander suppression. *Plant Biotechnol J*. 2011;9(9):982-9.

****An example of a plant-based vaccine targeting asthma, evaluated at the preclinical level**

[51] Li C, Jiang Y, Guo W, et al. Production of a chimeric allergen derived from the major allergen group 1 of house dust mite species in *Nicotiana benthamiana*. *Hum Immunol*. 2013;74(5):531-7.

[52] Tuomilehto, J. The emerging global epidemic of type 1 diabetes. *Curr Diab Rep* 2013; 13(6):795–804.

[53] Maahs DM, West NA, Lawrence JM, et al. Epidemiology of type 1 diabetes. *Endocrinol Metab Clin North Am*. 2010;39:481-497.

[54] D'Angeli MA, Merzon E, Valbuena LF, et al. Environmental factors associated with childhood-onset type 1 diabetes mellitus: an exploration of the hygiene and overload hypotheses. *Arch Pediatr Adolesc Med*. 2010;164:732-738.

[55] Wilkin TJ. The accelerator hypothesis: a review of the evidence for insulin resistance as the basis for type I as well as type II diabetes. *Int J Obes*. 2009;33:716-726.

[56] Ferrannini E, Mari A, Nofrate V, et al. Progression to diabetes in relatives of type 1 diabetic patients: mechanisms and mode of onset. *Diabetes* 2010;59:679-685.

[57] Gianani R, Eisenbarth GS. The stages of type 1A diabetes: 2005. *Immunol Rev* 2005; 204:232-49.

[58] Lee AS, Ghoreishi M, Cheng WK, et al. Toll-like receptor 7 stimulation promotes autoimmune diabetes in the NOD mouse. *Diabetologia* 2011;54(6):1407-1416.

[59] Lang KS, Recher M, Junt T, et al. Toll-like receptor engagement converts T-cell autoreactivity into overt autoimmune disease. *Nat Med*. 2005;11(2):138-145.

[60] Zhang Y, Lee AS, Shameli A, et al. TLR9 blockade inhibits activation of diabetogenic CD8+ T cells and delays autoimmune diabetes. *J Immunol*. 2010;184(10):5645-5653.

[61] Hu C, Ding H, Li Y, et al. NLRP3 deficiency protects from type 1 diabetes through the regulation of chemotaxis into the pancreatic islets. *Proc Natl Acad Sci USA*. 2015;112(36):11318-11323.

[62] Wen L, Ley RE, Volchkov PY, et al. Innate immunity and intestinal microbiota in the development of Type 1 diabetes. *Nature*. 2008;455(7216):1109-1113.

[63] Uno S, Imagawa A, Okita K, et al. Macrophages and dendritic cells infiltrating islets with or without beta cells produce tumour necrosis factor-alpha in patients with recent-onset type 1 diabetes. *Diabetologia* 2007;50(3):596-601.

[64] Tai N, Wong FS, Wen L. The role of the innate immune system in destruction of pancreatic beta cells in NOD mice and humans with type I diabetes. *J Autoimmun*. 2016;71:26-34.

[65] Control TD, Group CTR, Nathan DM, et al. The effect of intensive treatment of diabetes on the development and progression of long-term complications in insulin-dependent diabetes mellitus. *N Engl J Med*. 1993;329(14):977-86.

[66] Arakawa T, Yu J, Chong DK, et al. A plant based-cholera toxin B subunit insulin fusion protein protects against the development of autoimmune diabetes. *Nat Biotechnol*. 2016;16(10):934-8.

****Described a vaccine targeting autoimmune diabetes, evaluated at the preclinical level**

[67] Porceddu A, Falorni A, Ferradini N, et al. Transgenic plants expressing human glutamic acid decarboxylase(GAD65), a major autoantigen in insulin-dependent diabetes mellitus. *Mol Breed*. 1999;5(6):553-560.

[68] Avesani L, Falorni A, Tornielli GB, et al. Improved in planta expression of the human islet autoantigen glutamic acid decarboxylase (GAD65). *Transgenic Res*. 2003;12(2):203-12.

[69] Avesani L, Vitale A, Pedrazzini E, et al. Recombinant human GAD65 accumulates to high levels in transgenic tobacco plants when expressed as an enzymatically inactive mutant. *Plant Biotechnol J*. 2010;8(8):862-72.

[70] Li D, O'Leary J, Huang Y, et al. Expression of cholera toxin B subunit and the B chain of human insulin as a fusion protein in transgenic tobacco plants. *Plant Cell Rep*. 2006;25(5):417-24.

[71] Ma S, Huang Y, Yin Z, et al. Induction of oral tolerance to prevent diabetes with transgenic plants requires glutamic acid decarboxylase (GAD) and IL-4. *Proc Natl Acad Sci USA*. 2004;101(15):5680-5.

****An example of a vaccine targeting autoimmune diabetes, evaluated at the preclinical level**

[72] Merlin M, Gecchele E, Arcalis E. et al. Enhanced GAD65 production in plants using the MagnICON transient expression system: Optimization of upstream production and downstream processing. *Biotechnol J*. 2016;11(4):542-53.

****An example of a vaccine targeting autoimmune diabetes, in which production process is optimized**

[73] Smolen JS, Aletaha D, Redlich K. The pathogenesis of rheumatoid arthritis: new insights from old clinical data? *Nat Rev Rheumatol*. 2012;8(4):235-43.

[74] Arend WP, Firestein GS. Pre-rheumatoid arthritis: predisposition and transition to clinical synovitis. *Nat Rev Rheumatol*. 2012;8(10):573-86.

[75] Firestein GS. Evolving concepts of rheumatoid arthritis. *Nature*. 2003;423(6937):356-61.

[76] McInnes IB, Schett G. Pathogenetic insights from the treatment of rheumatoid arthritis. *Lancet*. 2017;389(10086):2328-2337.

[77] Ohnishi Y, Tsutsumi A, Matsumoto I, et al. Altered peptide ligands control type II collagen-reactive T cells from rheumatoid arthritis patients. *Mod Rheumatol*. 2006;16(4):226-8.

[78] Lizuka M, Wakasa Y, Tsuboi H, et al. Suppression of collagen-induced arthritis by oral administration of transgenic rice seeds expressing altered peptide ligands of type II collagen. *Plant Biotechnol J*. 2014;12(8):1143-52.

****A report on the therapeutic potential of a plant-based vaccine targeting arthritis, evaluated at the preclinical level**

[79] Lizuka M, Wakasa Y, Tsuboi H, et al. Prophylactic effect of the oral administration of transgenic rice seeds containing altered peptide ligands of type II collagen on rheumatoid arthritis. *Biosci Biotechnol Biochem*. 2014;78(10):1662-8.

****A report on the prophylactic potential of a plant-based vaccine targeting arthritis, evaluated at the preclinical level**

[80] Hansson C, Schön K, Kalbina I, et al. Feeding transgenic plants that express a tolerogenic fusion protein effectively protects against arthritis. *Plant Biotechnol J*. 2016;14(4):1106-15.

****Describes the preclinical evaluation of a plant-based vaccine targeting arthritis, evaluated at the preclinical level**

[81] Hirota T, Tsuboi H, Iizuka-Koga M, et al. Suppression of glucose-6-phosphate-isomerase induced arthritis by oral administration of transgenic rice seeds expressing altered peptide ligands of glucose-6-phosphate-isomerase. *Mod Rheumatol*. 2017;27(3):457-465.

****Describes the preclinical evaluation of a plant-based vaccine targeting arthritis, evaluated at the preclinical level**

[82] Mannucci PM, Tuddenham EG. The hemophilias--from royal genes to gene therapy. *N Engl J Med*. 2001;344(23):1773-1779.

[83] Bolton-Maggs PH, Pasi KJ. Haemophilias A and B. *Lancet*. 2003;361(9371):1801-1809.

[84] Mannucci PM, and Franchini M. Is haemophilia B less severe than haemophilia A? *Haemophilia* 2013;19(4):499-502.

[85] Terada K, Yagi Y, Niizuma T, et al. Is oral tolerance therapy possible for haemophilia with inhibitors? *Vox Sang*. 2001;80(1):61-62.

[86] Verma D, Moghimi B, LoDuca PA, et al. Oral delivery of bioencapsulated coagulation factor IX prevents inhibitor formation and fatal anaphylaxis in hemophilia B mice. *Proc Natl Acad Sci USA*. 2010;107(15):7101-7106.

****A case of a plant-based vaccine targeting the induction of blocking antibodies, evaluated at the preclinical level**

[87] Wang X, Su J, Sherman A et al. Plant-based oral tolerance to hemophilia therapy employs a complex immune regulatory response including LAP+CD4+ T cells. *Blood*. 2015;125(15):2418-2427.

****A mechanistic study on a plant-based vaccine targeting the induction of blocking antibodies**

[88] Su, J, Zhu L, Sherman A, et al. Low cost industrial production of coagulation factor IX bioencapsulated in lettuce cells for oral tolerance induction in hemophilia B. *Biomaterials* 2015;70:84-93.

****Describes the implementation of the industrial production of a plant-based vaccine targeting the induction of blocking antibodies**

[89] Herzog RW, Nichols TC, Su J, et al. Oral Tolerance Induction in Hemophilia B Dogs Fed with Transplastomic Lettuce. *Mol Ther*. 2017;25(2):512-522.

****An example of a plant-based vaccine targeting the induction of blocking antibodies, evaluated in an advanced animal model**

[90] Sherman A, Su J, Lin S, et al. Suppression of inhibitor formation against FVIII in a murine model of hemophilia A by oral delivery of antigens bioencapsulated in plant cells. *Blood*. 2014;124(10):1659-1668.

****A case of a plant-based vaccine targeting the induction of blocking antibodies, evaluated at the preclinical level**

[91] Chan AC1, Carter PJ. Therapeutic antibodies for autoimmunity and inflammation. *Nat Rev Immunol*. 2010;10(5):301-16.

[92] Galeotti C, Kaveri SV, Bayry J1. IVIG-mediated effector functions in autoimmune and inflammatory diseases. *Int Immunol*. 2017;29(11):491-498.

[93] Abbas AK, Trotta E, R Simeonov D, et al. Revisiting IL-2: Biology and therapeutic prospects. *Sci Immunol*. 2018;3(25):eaat1482.

[94] Bacher P, Scheffold A. The effect of regulatory T cells on tolerance to airborne allergens and allergen immunotherapy. *J Allergy Clin Immunol*. 2018;142(6):1697-1709.

[95] Pozsgay J, Szekanecz Z, Sármay G. Antigen-specific immunotherapies in rheumatic diseases. *Nat Rev Rheumatol*. 2017;13(9):525-537.

[96] Wraith DC. The Future of Immunotherapy: A 20-Year Perspective. *Front Immunol*. 2017;8:1668.

[97] Su J, Zhu L, Sherman A, Wang X, Lin S, Kamesh A, Norikane JH, Streatfield SJ, Herzog RW, Daniell H. Low cost industrial production of coagulation factor IX bioencapsulated in lettuce cells for oral tolerance induction in hemophilia B. *Biomaterials*. 2015;70:84-93.

[98] Lakshmi PS, Verma D, Yang X. Low cost tuberculosis vaccine antigens in capsules: expression in chloroplasts, bio-encapsulation, stability and functional evaluation in vitro. *PLoS One*. 2013;8(1):e54708.

[99] Kwon KC, Daniell H. Low-cost oral delivery of protein drugs bioencapsulated in plant cells. *Plant Biotechnol J*. 2015;13(8):1017-1022.

***A review on the potential of plant cells to serve as oral delivery vehicles**

[100] Peyret H, Lomonossoff GP. When plant virology met *Agrobacterium*: the rise of the deconstructed clones. *Plant Biotechnol J*. 2015;13(8):1121-35.

[101] Bendandi M, Marillonnet S, Kandzia R, et al. Rapid, high-yield production in plants of individualized idiotype vaccines for non-Hodgkin's lymphoma. *Ann Oncol*. 2010;21(12):2420-7.

[102] Klimyuk V, Pogue G, Herz S, et al. Production of recombinant antigens and antibodies in *Nicotiana benthamiana* using 'magniffection' technology: GMP-compliant facilities for small- and large-scale manufacturing. *Curr Top Microbiol Immunol*. 2010;375:127-154.

[103] Dugdale B, Mortimer CL, Kato M, et al. In plant activation: an inducible, hyperexpression platform for recombinant protein production in plants. *Plant Cell*. 2013;25(7):2429-2443.

[104] Arntzen C. Plant-made pharmaceuticals: from 'Edible Vaccines' to Ebola therapeutics. *Plant Biotechnol J*. 2015;13(8):1013-1016.

[105] Tusé D, Tu T, McDonald KA. Manufacturing economics of plant-made biologics: case studies in therapeutic and industrial enzymes. *Biomed Res Int*. 2014;2014:256135.

[106] Ritacco FV, Wu Y, Khetan A. Cell culture media for recombinant protein expression in Chinese hamster ovary (CHO) cells: History, key components, and optimization strategies. *Biotechnol Prog*. 2018;34(6):1407-1426.

[107] Sanchez-Garcia L, Martin L, Mangues R, Ferrer-Miralles N, et al. Recombinant pharmaceuticals from microbial cells: a 2015 update. *Microb Cell Fact*. 2016;15:33.

[108] Sivakumar G., Ed. (2017) *New Insights into Cell Culture Technology*. pp. 43–97, InTech, Rijeka, Croatia.

[109] Shaaltiel Y, Bartfeld D, Hashmueli S, et al. Production of glucocerebrosidase with terminal mannose glycans for enzyme replacement therapy of Gaucher's disease using a plant cell system. *Plant Biotechnol J*. 2007;5(5):579-590.

***A study on the first plant-made biopharmaceutical approved for human use**

[110] Shaaltiel Y, Gingis-Velitski S, Tzaban S, et al. Plant-based oral delivery of β -glucocerebrosidase as an enzyme replacement therapy for Gaucher's disease. *Plant Biotechnol J*. 2007;13(8):1033-1040.

[111] Qiu X, Wong G, Audet J, et al. Reversion of advanced Ebola virus disease in nonhuman primates with ZMapp. *Nature*. 2014;514(7520):47-53.

[112] Yusibov V, Kushnir N, Streatfield S. Antibody Production in Plants and Green Algae. *Annu Rev Plant Biol*. 2016;67:669-701.

[113] Ruhlman T, Ahangari R, Devine A, Samsam M, Daniell H. Expression of cholera toxin B-proinsulin fusion protein in lettuce and tobacco chloroplasts: oral administration protects

against development of insulinitis in non-obese diabetic mice. *Plant Biotechnol J.* 2016;5:495–510.

[114] Boyhan D, Daniell H. Low-cost production of proinsulin in tobacco and lettuce chloroplasts for injectable or oral delivery of functional insulin and C-peptide. *Plant Biotechnol J.* 2011;9(5):585-98.

Accepted Manuscript

Table 1. Summary of the plant-based vaccines targeting allergies and autoimmune/inflammatory disorders.

Target disorder	Target antigen	Expression approach/crop	Main Findings	References
Allergy to Birch pollen	Bet v 1	Viral transfection/ <i>N. benthamiana</i>	Expression levels of 200 µg/g fw; Bet v 1 retained antigenicity and immunogenicity.	[34]
Allergy to Japanese cedar pollen	Cry j 1	<i>Agrobacterium</i> stable transformation/rice seeds	Expression levels of 1.6 µg/mg TP in seeds; IgE antibodies from allergic patients did not recognize the recombinant polypeptide.	[35]
Allergy to Japanese cedar pollen	Cry j 1 and Cry j 2 (7Crp)	<i>Agrobacterium</i> stable transformation/rice seeds	Expression levels about 60 µg/grain; 30% of reduction in IgE antibodies levels in comparison to WT rice.	[36]
Allergy to Japanese cedar pollen	Cry j 1 and Cry j 2 (7Crp)	<i>Agrobacterium</i> stable transformation/ rice seeds	Expression levels of 0.5% of TP in seeds; oral immunization in mice induced the reduction of clinical symptoms mediated by the abrogation of IgE antibodies and T CD4 ⁺ proliferative responses.	[37]
Allergy to Japanese cedar pollen	Cry j 1 and Cry j 2 (7Crp)	<i>Agrobacterium</i> stable transformation / rice seeds	Stable integration of the transgene was proven in T7 rice plants; the content of biomolecules has not been modified in transgenic plants. In addition, posttranscriptional modifications such as N-glycosylation were not observed.	[38]
Allergy to Japanese cedar pollen	Cry j 1 and Cry j 2 (7Crp)	<i>Agrobacterium</i> stable transformation/ rice seeds	The accumulation of 7Crp in ER-derived PB-I reached up to 60 µg/grain, morphological alterations of protein bodies were observed.	[39]
Allergy to Japanese cedar pollen	Cry j 1 and Cry j 2 (3Crp, 3Crp-7Crp)	<i>Agrobacterium</i> stable transformation with direction of proteins to protein bodies of ER or vacuole/ rice seeds	The accumulation of 3Crp or 3Crp-7Crp peptides in protein bodies improved the delivery system, degradation of peptides was slower, and the necessary doses to abrogate allergy were 20-fold lesser than the synthetic peptides.	[40]
Airway inflammation by mite allergen	Der p2	<i>Agrobacterium</i> stable transformation/ <i>Nicotiana tabacum</i>	The accumulation of Der p2 reached up to 0.5% TSP, the oral tolerance scheme in mice induced the abrogation of airway inflammation symptoms mediated by the increase of regulatory T cells and the reduction of IgE and IgG1 antibodies, IL-4, IL-5, IL-13, and eotaxin.	[41]
Bronchial asthma by mite allergen	Der p1 ₄₅₋₁₄₅	<i>Agrobacterium</i> stable transformation/rice seeds	The Der p1 peptide accumulated in PB-I protein body with N-glycosylation as posttranscriptional modification. The induced oral tolerance abrogated the IgE antibodies and Th2 cellular responses.	[42]
Allergy to Japanese cypress pollen and Japanese cedar pollen	Chao 1, Chao 2, Cry j1 and Cry j2 (3Crp, 6Chao, 7Crp-3Crp-6Chao)	<i>Agrobacterium</i> stable transformation/rice seeds	The accumulation levels of polypeptides reached up to 2.75 mg/g seed, the accumulation mainly occurred in ER-derived PB-I bodies, and 30 min were required for the complete in vitro digestion with pepsin.	[30]
Diabetes	CTB-insulin fusion	<i>Agrobacterium</i> stable transformation/ potato plants	Insulinitis reduction and diabetes progression diminished in NOD mice (non-obese diabetic mice)	[66]
Autoimmune Type 1 diabetes mellitus (T1DM)	GAD65	<i>Agrobacterium</i> stable transformation/ carrot and tobacco	GAD65 protein was correctly expressed and folded; it accumulated up to 0.01 and 0.04% TSP in tobacco and carrot, respectively.	[67]
Autoimmune Type	hGAD67/65	<i>Agrobacterium</i> stable	Stable transformation produced 0.19	[68]

1 diabetes mellitus (T1DM)	and hGAD65	transformation/ Tobacco, cv. Lonibow and, Transient transformation/ <i>N. benthamiana</i>	% TSP and 2.2 % by transient transformation.	
Autoimmune Type 1 diabetes mellitus (T1DM)	IL-4 and GAD65	<i>Agrobacterium</i> stable transformation/ Tobacco	Immunization with IL-4 plus GAD65 reduced insulinitis and protected NOD mice from diabetes.	[71]
Autoimmune Type 1 diabetes mellitus (T1DM)	CTB-InsB3	<i>Agrobacterium</i> stable transformation/ Tobacco	The expression yield reached up to 0.11% TSP and the fusion protein conserved the GM1-ganglioside receptor binding specificity.	[72]
Autoimmune Type 1 diabetes mellitus (T1DM)	CTB-Pins	Chloroplast trans-formation / tobacco and lettuce	Immunization with CTB-Pins resulted in decreased lymphocyte migration in pancreas, β -cells conservation in the pancreatic islets, and low glucose levels when compared to controls.	[113]
Autoimmune Type 1 diabetes mellitus (T1DM)	GAD65, GAD65mut and GAD67/65mut	<i>Agrobacterium</i> stable transformation/ Tobacco	GAD65mut preserved the immunogenic properties of GAD65 and accumulated up to 2.2 % TSP due to a highly active protein synthesis. Targeting to ER did not increase protein accumulation.	[69]
Autoimmune Type 1 diabetes mellitus (T1DM)	CTB-PFx3	Chloroplast transformation / tobacco and lettuce	Immunizations with transgenic material significantly reduced blood glucose levels when compared to control group; with similar levels to using commercially available insulin.	[114]
Autoimmune Type 1 diabetes mellitus (T1DM)	GAD65mut, Δ 38GAD65mut, and Δ 87GAD65mut	<i>Agrobacterium</i> stable transformation and agroinfiltration transient transformation/ tobacco	GAD65 expression was optimized using the truncated version of GAD65 lacking the first 87 amino acids and using MacnICON system.	[72]
Hemophilia B	CTB-FIX and CTB-FFIX	Stable transformation of the chloroplast genome by biolistics/ <i>Nicotiana tabacum</i>	Expression levels ranged 0.19-3.8% TSP (0.4 mg/g of leaf tissue) and suppression of F.IX inhibitors and anaphylaxis in C3H/HeJ F9-/- mice	[86]
	CTB-FFIX	Stable biolistic transformation of leaf chloroplast genome/ <i>Lactuca sativa</i>	Mechanisms involved in the development of induced tolerance, a latency-associated peptide expressing CD4+ regulatory T cells (CD4+ CD25-LAP+ cells with positively regulated IL-10 and expression of TGF- β). Conventional CD4+ CD25+ regulatory T cells systematically suppressed anti-FIX responses	[87]
			Expression levels of ~1 mg per gram of dry tissue, stable up to two years at room temperature and prevention of anaphylactic reactions and inhibition of h.F.IX in C3H/HeJ F9-/- mice	[88]
			Stable expression levels up to 30 months of ~1 mg per gram of dry tissue and suppression of anaphylactic and inhibition of h.F.IX in a larger animal model (hemophilia B dogs)	[89]
Hemophilia A	CTB-HC and CTB-C2	Stable transformation of the chloroplast genome by biolistics/ <i>Nicotiana tabacum</i>	Expression levels of 80 and 370 μ g/g in fresh leaves, suppression of F.VIII inhibitors and anaphylaxis in C57BL6/129 F8e16-/- mice and induction of CD4+ CD25+ and CD4+ CD25- T cells that potently suppressed anti-FVIII formation	[90]

Figure legends

Fig 1. Schematic representation of the mechanisms proposed for oral plant-based vaccines capable of inducing tolerance. The plant-based vaccine is administered orally. 1. Once in the gut, the antigen can be captured by the intestinal mucosa through microfold (M) cells that exist in the follicle-associated epithelium of the Peyer's patches. The antigen is deposited in antigen-presenting cells (APCs); these cells process it and expose it in their membrane. 2. Primed APCs travel through lymphoid circulation to secondary lymphoid organs; they interact with Th0 cells activating them and polarizing their activation towards T cells CD4+CD25+FOXP3+ (Tregs cells). 3. Tregs cells travel through systemic circulation avoiding immune activation against allergens and replacement biopharmaceuticals. 4. Finally, Treg cells can decrease the number of active elements of the immune system in diseases such as diabetes or asthma.

Figures

Fig. 1.

