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REVIEW

Plant-based vaccines against respiratory diseases: current status and future prospects

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ABSTRACT

Introduction: Respiratory infections have an enormous, worldwide epidemiologic impact on humans and animals. Among the prophylactic measures, vaccination has the potential to neutralize this impact. New technologies for vaccine production and delivery are of importance in this field since they offer the potential to develop new immunization approaches overriding the current limitations that comprise high cost, safety issues, and limited efficacy.

Areas covered: In the present review, the state of the art in developing plant-based vaccines against respiratory diseases is presented. The review was based on the analysis of current biomedical literature.

Expert commentary: Preclinical and clinical evaluations of several vaccine candidates against influenza, tuberculosis, respiratory syncytial virus, pneumonia, anthrax and asthma are discussed and placed in perspective.

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1. Introduction

The concept of plant-based vaccines consists in using plants as antigen biofactories following genetic engineering procedures. The antigen is subsequently purified from plant material to formulate parenteral vaccines or the plant material can be freeze dried for direct use in the formulation of oral vaccines [1]. Plants offer several advantages since their biomass can be generated at low cost in a large scale using low-cost bioreactors or greenhouses employing materials that are cheaper when compared to conventional fermentation systems or mammalian cell culture systems; in addition, when oral delivery is pursued no purification of the antigen from the plant biomass is needed, which represents huge savings in the production process. In addition, plants do not replicate human pathogens, and have the machinery to produce complex antigens. These features overcome the limitations that are inherent to the conventional systems based on bacteria, insect cells, or mammalian cells; where the presence of endotoxins, risk of viral contamination, or high costs exist [2]. In addition, there is the possibility of using plant biomass as the delivery vehicle for oral vaccines, which represents the opportunity to formulate vaccines at very low costs by freeze drying the biomass and making tablets instead of using extensive and expensive purification processes [3,4].

The state of the art in plant-based vaccines includes several accomplishments: (i) this technology is currently of great interest for the scientific community with a sustained increase in the scientific reports regarding plant-based vaccine prototypes, (ii) some plant-based vaccines have been evaluated in pilot or clinical trials [5–8], (iii) some human vaccine prototypes are currently under clinical evaluation [9], and (iv)

industrial processes for GMP production of plant-based vaccines have been implemented [3,10,11]. In addition, a non-vaccine plant-made biopharmaceutical (the glucocerebrosidase enzyme) is currently commercialized for the treatment of Gaucher's disease [12] and a vaccine for poultry (against *Newcastle disease virus*) was approved by the United States Department of Agriculture (USDA) [13].

Among the groups of target diseases for which plant-based vaccine technology can be applied to promote human health around the world, respiratory diseases deserve special attention. For instance, approximately 200,000 people die from pulmonary infections in the USA each year. Influenza, pneumonia, and lung disease exacerbated by pulmonary infection, are the most frequent conditions responsible for the epidemiological impact of respiratory diseases. In addition, respiratory diseases such as asthma affect 300 million people worldwide [14]. Given this relevance, the present review contains an updated outlook on the development of plant-based vaccines targeting respiratory diseases.

2. Modalities for the expression of antigens in plants

The technologies to express heterologous proteins in the plant cell can be categorized in three modalities: chloroplast-based expression (transplastomic technologies), transient expression (viral or nonreplicative vectors), and stable nuclear expression. Chloroplast expression relies on the site-specific integration of the heterologous DNA into the circular chloroplast genome (plastome). In contrast to nuclear expression, which is influenced by position effects, copy number, and sites of insertion; the plastome is present in a high copy number

and no position effects or silencing mechanisms are known to date for this system [15]. All these factors lead to substantially higher yields when compared to stable nuclear transformation. In addition, the maternal inheritance of the plastome adds another advantage to this technology, since increased biosafety is attained due to the lack of undesired gene flow via pollen derived from the fact that the plastome is maternally inherited [16]. A drawback of the transplastomic technologies consists of the long time required for generating transplastomic lines and the lack of reproducible transformation protocols for many relevant plant species.

The stable insertion of the transgene is the conventional approach for heterologous protein expression in plants. However, long times are required to obtain stable transformed plant lines. The expression vectors based on plant virus elements can also be used to express nuclear-encoded antigens following transient expression, which implies a short time to achieve expression. Some vector designs consist of 'deconstructed viruses,' which are delivered by *Agrobacterium* in the form of T-DNAs carrying RNA/DNA viral replicons that once in the cell induce massive RNA and protein production. This approach provides fast production, easy scalability, robust yields, and available industrial processes under GMP. Such attributes offer enormous advantages for most of the cases following nuclear stable expression. However, no seed stocks can be generated when viral-mediated expression is followed. The main viruses that have served as the source of elements for designing vectors include RNA viruses such as the *Tobacco mosaic virus* (TMV), *Potato virus X* (PVX), and *Cowpea mosaic virus* (CPMV); as well as DNA geminivirus such as the *Bean yellow dwarf virus* [17]. Transient expression can also be performed using nonreplicative vectors delivered by agroinfiltration [18].

When nuclear expression is followed, the antigenic protein can be localized in specific cellular compartments, e.g. the endoplasmic reticulum (ER), protein bodies, or chloroplasts [19]. In particular, the ER is attractive because of the presence of the glycosylation machinery among several other post-translational modifications that occur in this organelle as well as a low protease content. Other approaches comprise the localization of the antigen in protein bodies or oil bodies, which can increase yields and facilitate purification [20,21]. Moreover, transient expression systems also offer possibility to target protein of interest into a specific compartment. Overall, all these expression methodologies have been applied in the development of plant-based vaccines against respiratory diseases; with no strong bias toward a specific expression modality.

3. Current developments of plant-made vaccines targeting respiratory pathologies

3.1. Influenza virus

Influenza is an acute viral infection caused by the influenza virus. There are three types of seasonal influenza viruses: A, B, and C. Based on the combinations of several surface proteins, type A influenza viruses can be classified into subtypes. The influenza A (H1N1) and A (H3N2) subtypes are currently

circulating in humans. The most prominent influenza B viruses include B/Yamagata and B/Victoria. Type C influenza occurs less frequently than A and B. For this reason, only influenza A and B viruses are included in seasonal influenza vaccines. The annual rate of infection is estimated at 5–10% in adults and 20–30% in children. The most affected groups are infants, elderly, and chronically ill patients. Annual epidemics are estimated at about 3–5 million cases of severe illness with 250,000–500,000 deaths [22].

Safe and effective vaccines are available and have been used for more than 60 years to prevent seasonal influenza. In healthy adults, the inactivated influenza vaccine can provide reasonable protection, although not much protection is required. In children less than 2-year old and the elderly, the protection seemed to fail; the reason could be that the immune system function is limited and the humoral immune response elicited by the inactivated vaccine may be insufficient and with a rapid decay. In industrialized countries, most deaths occur among 65-year-old individuals in whom the immune system may not adequately react to vaccination; therefore, new adjuvants are under exploration to improve efficacy in the elderly, e.g. squalene and tocopherol oil-in-water emulsion-based adjuvant system together with the immunostimulant monophosphoryl lipid A [23]. The live attenuated and adjuvanted vaccines are the most promising for this group, although the live attenuated vaccine has more side effects [24]. In any case, an early vaccine availability is a key factor in reducing the pandemic influenza morbidity and mortality.

The current platforms for influenza vaccine production are prominently egg-based viral replications for the production of inactivated (killed) and live attenuated vaccines. The same application is also performed in mammalian cell lines. A recombinant version based on the production of a specific antigen in genetically engineered insect cells is also applied for the production of influenza vaccines [25]. These platforms require, in general, animal-derived products, costly purification processes, and the handling of virus that demand high biosafety facilities. The plant-based platforms offer low production costs, absence of animal pathogens, and the absence of virus in the production process.

The development of plant-based vaccines against influenza is ongoing, with several candidates being evaluated at the preclinical level with some of them under evaluation in phase I clinical trials. The development of subunit vaccines against influenza is primarily based in the use of the hemagglutinin (HA), a surface glycoprotein with a key role in the infectivity of the influenza virus, and the M1 protein; a matrix protein located in the viral core that constitutes the most abundant structural protein. The earlier initial studies in this field comprised the expression of HA and NA using a launch vector for transient expression [26,27]. Subsequently, D'Aoust et al. [28] reported in 2008 the production of enveloped influenza virus-like particles (VLPs) in plants.

In the case of HA, it is initially synthesized in the infected cell as a single polypeptide chain (HA0) that is subsequently processed to yield two subunits, HA1 and HA2. The expression of HA1 from seasonal strain A/New Caledonia/20/99 (H1N1) and M1 protein from influenza matrix protein strain A/Puerto

Rico/8/34 (AGL1/750) was conducted to evaluate if M1 is required for the formation of VLPs in the plant cell. The expression of the HA gene of the H5 subtype from A/Indonesia/5/05 (H5N1) only, with its native signal peptide and transmembrane (TM) domain, allowed the viral protein budding from the plasma membrane forming VLPs. This finding indicated that M1 is not required for the formation of VLPs. Six days post-infiltration, the HA expression level in leaf tissue was 50 mg/kg fresh weight. The successfully produced VLPs resembling the influenza virions in size and morphology were used to immunize BALB/c, intramuscularly (i.m.), twice at 3-week intervals using a formulation containing 0.1–12 µg of H5N1VLPs along with alum. The production of antibodies that inhibited the viruses from agglutinating erythrocytes at high dilutions was proven. Remarkably, the immunogenic activity of this plant vaccine was sufficient to confer protection against a challenge with the A/Vietnam/1194/04 strain in mice immunized even with low antigen doses (0.5 µg). This pioneering study opened a path for the exploration of the large-scale production of highly immunogenic VLPs-based plant-made influenza vaccines with an estimated potential to yield 1500 vaccine doses for humans per kilogram of infiltrated leaves [28].

Landry et al. [29] reported additional developments for this vaccine. For preclinical assessment, the vaccine consisting of varying doses of pure VLPs (0.7, 1.8, 3.7, or 11.0 µg) was i.m. administered along with Alhydrogel to ferrets. The plant-made vaccine induced detectable hemagglutination inhibition (HI) antibodies at all tested doses, with the highest response in the group receiving 11 µg doses. After the second dose, 100% of the ferrets had detectable HI antibodies surviving to a lethal viral challenge. Subsequently, the vaccine was administered to humans in a phase I clinical trial at doses of 5, 10, or 20 µg of HA. In the 20-µg group, most of the subjects had antibodies able to prevent cell infection by live viruses and promote complement-mediated hemolysis induced by influenza antigen–antibody complexes. Despite the presence of plant-specific glycans in this vaccine, which are not present in mammals (α-1,3-fucose and β-1,2-xylose residues and terminal N-acetylglucosamine), its i.m. administration did not induce IgG or IgE responses to plant glycans, which accounts for the safety of this recombinant vaccine. *In vitro* neutralization studies and *in vivo* challenges in ferrets demonstrated cross-clade protection induced by this candidate vaccine. In addition, in the clinical studies, a 50% protection index was achieved by the 20-µg dose group. This report constitutes the first phase I clinical trial for a plant-based influenza vaccine, which represents a milestone that will lead to further trials to achieve the approval and commercialization of the first plant-made vaccine for human use.

In another study, VLPs formed by the expression of the native HAs from the strains A/New Caledonia/7/2009 (H1N1) or A/Indonesia/5/05 (H5N1) were transiently expressed in *Nicotiana benthamiana* detecting HA1, HA2, as well as dimers of HA0 (precursor) [30]. The N-terminal sequencing demonstrated that the signal peptide was properly cleaved in the host. Although this study did not present any immunogenicity assessment, the ability of plants to mainly produce complex mature N-glycans for H1 and H5 antigens carrying core α (1,3)-

glucose, β(1,2)-xylose epitopes as well as Lewis a (Le^a) epitope was demonstrated; moreover, this analysis also showed that the Asn11 of the N10NST13 sequence is the only glycosylation site processed in the mature HA [30]. A further characterization of sphingolipids identified the glucosylceramide d18:2, h16:0 as the most abundant in the plant-made VLPs. The data indicate an attractive biosynthetic capacity of the plant cell for the production of mature and glycosylated forms of HA.

Improvements for the transient VLPs production platforms were subsequently achieved by modifying the HA with plant sequences. The substitution of the HA native signal peptide with the signal peptide of calreticulin (CA) from *Nicotiana tabacum*, as well as replacing the TM and cytosolic tail (CT) domains by heterologous sequences, led to an improvement in expression efficiency for HA from the A/California/04/09 strain (HAC). The immunogenicity assessment in mice revealed that the i.m. immunization with doses in the 0.02–15 µg range induced high humoral responses. A single administration of HAC-VLPs at 15 or 3 µg in the presence of Alhydrogel elicited HI antibody titers of ≥1:40 in 90% and 50% of the animals, respectively. These titers were significantly enhanced after the second immunization. HAC-VLPs induced balanced Th1/Th2 responses, in contrast to the HAC protein in the monomeric form that showed lower potency [31].

Further efforts in this field consisted in the expression of HAs from A/Brisbane/59/07, A/Brisbane/10/07, B/Florida/4/06, and A/California/04/09, abbreviated as HAB1(H1), HAB1(H3), HAF1(B), and HAC1; respectively. Transiently, *N. benthamiana*-expressed proteins were obtained at levels of 400–1300 mg/kg of fresh leaf tissue. BALB/c mice were used to estimate the immunogenic properties of the plant-made vaccines following two immunization schemes: (i) three s.c. immunizations at 2-week intervals with HAB1(H1), HAB1(H3), or HAF1(B) using 60-, 30-, or 15-µg doses along with 10 µg of Quil A as adjuvant; (ii) two immunizations at a 3-week interval (days 0 and 21) with HAC1 administered either subcutaneously (tested doses of 30, 15, or 5 µg with 10 µg of Quil A) or i.m. (tested dose of 30 µg along with the adjuvant Alhydrogel). All animals immunized with HAB1(H1), HAB1(H3), or HAF1(B) showed serum HA-specific IgG responses that increased after the subsequent boosting. Serum HI antibody responses in mice s.c. immunized with HAC1 and Quil A were measured using A/California/07/09 or A/Texas/5/09, two novel H1N1 influenza A virus strains, observing positive results. Test mice i.m. immunized with a 30 µg dose of HAC1 with Alhydrogel also showed serum HI antibody production at 10 days after boosting. Although the mean titer was lower when compared to the mice immunized s.c. with HAC1 and Quil A, the values were ≥1:40 in all animals at 10 days post-boosting and reached the level of 1:718.4 ± 350.5 at 21 days after boosting. Therefore, at this stage, Alhydrogel was identified as a promising adjuvant for the i.m. administered vaccine [32]. In terms of safety of this vaccine, the lack of humoral responses against the His tag and the KDEL sequence or adverse responses associated to plant glycans have been detected in mice treated with the HAC1 vaccine [33].

The race for clinical evaluation of influenza plant-based vaccines had advances in 2014 with the phase I evaluation

of the HAC1 vaccine (His-tagged HA monomer from A/California/04/2009 (H1N1) influenza virus fused to the ER retention signal, KDEL). HAC1 was i.m. administered, whereas the licensed Influenza A (H1N1) 2009 monovalent inactivated influenza virus vaccine with a single i.m. dose and no adjuvant was used as control. Groups of 10 volunteers received two doses of the vaccine formulation on days 1 and 22, a placebo, or the active control. HAC1 was evaluated at doses of 15, 45, or 90 µg with or without adjuvant. Hemagglutination inhibition (HAI) and virus microneutralization (MN) antibody titers were subsequently evaluated, obtaining very positive outcomes: there was no dose-related toxicity, HAC1 induced seroconversion rates between 20% and 56% according to HAI and 50–80% according to MN; a humoral response enhancement was observed after the second immunization with HAC1 at the 90-µg dose (with no adjuvants) as the most efficient vaccine (78% according to HAI and 100% according to MN). The inclusion of Alhydrogel® as adjuvant was inefficient for this vaccine, which was in contrast with the previous observation made in the preclinical evaluation [34].

Interestingly, the mucosal immunization routes are also under exploration for the plant-made influenza antigens. In a combination of nanotechnology and molecular farming, a *N. benthamiana*-made H1N1 influenza virus A/California/04/09 (HAC1) was administered two times by the intratracheal (i.t.) route in several combinations comprising the use of a silica nanoparticle-based (SiO₂) drug delivery system and a mucosal adjuvant candidate, bis-(3',5')-cyclic dimeric guanosine monophosphate (c-di-GMP). HAC1 intraperitoneally with alum was used as the positive control. The mucosally administered vaccine induced systemic antibody responses at levels comparable to those induced by the parenteral vaccine. Remarkably, mucosal IgG and IgA were induced by the single-adjuvanted vaccine HAC1/c-di-GMP. A local T_H cell response was also induced by the c-di-GMP-vaccine. This seminal work could lead to new vaccines capable of inducing efficient immune responses (cellular and humoral) not only in the systemic compartment but in the site of entry of respiratory pathogens, the airways [35].

More recently, Major et al. [36], in contrast to the study of Neuhaus et al. [29], evaluated in mice and ferrets the protective efficacy of a c-di-GMP-adjuvanted plant-derived H5 influenza vaccine following intranasal (i.n.) immunization. The recombinant vaccine was constructed with amino acids 17–532 from the HA gene of the A/Indonesia/05/2005 (H5N1) virus and produced by transient expression as soluble recombinant protein. BALB/c mice were immunized i.n. with the plant-derived vaccine in the presence or absence of 5 µg of adjuvant at days 0 and 21. Three different doses of the vaccine were tested (15, 5, and 1.5 µg) leading to the protection of the animals from morbidity and mortality associated with a highly pathogenic avian influenza virus challenge.

3.2. Respiratory syncytial virus

The Respiratory syncytial virus (RSV) belongs to the Paramyxoviridae family and constitutes the main causative agent of the lower respiratory tract illness (LRI) in infants [37]. Over 30 million of new acute lower respiratory infection

episodes in children under five are caused by RSV per year, resulting in more than 3.4 million hospital admissions [38]. The prevalence of RSV in the elderly is also high. The immunity to natural RSV infection is partial but protective and CD8 + T cell responses are associated to immunoprotection [39].

Currently, no licensed vaccines for RSV have been achieved, and those further advances in vaccine development are required. The challenges in this field had mainly comprised adverse effects, including the fatal incidence of the formaline-inactivated (FI-RSV) vaccine occurred in the 1960s [40–42]. Currently, around 50 vaccine candidates are under development; in addition, monoclonal antibodies have also played a relevant role in RSV therapy.

The fusion (F) protein is the major RSV antigenic protein and was used to produce a tomato-based vaccine. The F-RSV gene was expressed in transgenic tomato plants under the control of either the constitutive CaMV 35S promoter or the fruit-specific E8 promoter. This promoter led to similar recombinant protein yields (12.68 ± 2.55 and 9.01 ± 1.87 µg/g fruit fresh weight for E8 and CaMV 35S promoters, respectively). The immunogenic potential of the tomato-made RSV-F protein was assessed in BALB/c mice fed with tomato fruits (3–4 g) five times during 28 days. Most of the mice showed significant ($p \leq 0.05$) serum IgG and IgA responses and produced anti-RSV-F antibodies, developing significant RSV-F-specific intestinal IgA titers. Mice i.m. boosted with an unspecified dose of RSV-inactivated antigen showed significantly ($p \leq 0.05$) higher titers of serum IgG2a and IgG2b (end point titer 1:11,000) compared to serum IgG1 (end point titer 1:8000). These results suggest that the transgenic-fruit-derived RSV-F antigen primed a mixed-type 1–2 T_H helper cell immune response, which was subsequently polarized toward the Th₁ type after the boost. No signs of adverse effects were observed in the mice treated with F-RSV [43].

Although the F protein is the most conserved and antigenic RSV target, the attachment protein (G) is capable of inducing immunoprotection in mice following immunization by i.n. or intraperitoneal (i.p.) routes [44]. Therefore, the G protein has also been the basis for plant-based vaccine development. Peptides from G protein of the human RSV A2 strain (NF1-RSV/172-187 and NF2-RSV/170-191) were separately fused to the Alfalfa mosaic virus (AIMV) coat protein (CP) and individually expressed in *N. tabacum* cv. Samsun NN plants. After virus infection, the plants accumulated an average of 0.5 mg of NF1-RSV or NF2-RSV per gram of fresh tissue, equivalent to 50 µg of pure RSV peptide. BALB/c mice were intraperitoneally immunized with three doses of the purified recombinant vaccine showing high levels of serum G protein-specific antibodies as well as protection against a challenge with RSV Long strain [45].

The continuation of the study was published in 2005 by Yusibov et al. [46] who constructed a fusion antigen called VMR-RSV, consisting of a 21-mer peptide representing amino acids 170–190 of the G protein fused to the AIMV CP. VMR-RSV was produced by transient expression in transgenic *N. tabacum* cv. Samsun NN plants expressing the AIMV P1 and P2 (P12) replicase genes. The antigenic properties of this vaccine were evaluated in human peripheral blood mononuclear cells (PBMCs) from five individuals with different HLA alleles; which

were treated with 10 µg of VMR-RSV and CD4+, CD8+, and CD56+ (NK) T cell responses were subsequently analyzed. A DCs analysis indicated that presentation by both classes I and II molecules as well as an expansion of IFN- γ -producing NK cells (CD56+) occurred. However, no immunologic memory was generated to VMR-RSV. An *in vivo* study demonstrated that nonhuman primates i.m. immunized were able to develop humoral and cellular responses. Higher and earlier T cell responses and antibody levels in the Rhesus were observed when compared to the Cynomolgus macaque. Interestingly, vigorous immune responses were generated without the use of any adjuvant.

The latest report on RSV plant-based vaccines found in the literature was published by Lau and Korban [47], in which the F protein was expressed in apple plants (*Malus domestica* Borkh. cv. Royal Gala) transformed via *Agrobacterium*. The levels of RSV-F antigen in different transgenic plants ranged from 0 to 20.1 µg/g of leaf tissue, with an overall average of 2.4 µg/g. Despite the observed expression levels, no immunogenicity tests were conducted.

3.3. *Streptococcus pneumoniae*

Pneumonia is the major cause of deaths in children, killed 935,000 children under 5-year old in 2003. Several agents can cause pneumonia including: *Streptococcus pneumoniae*, *Haemophilus influenzae* type B (Hib), RSV, and *Pneumocystis jiroveci* [48]. *Streptococcus pneumoniae* is a polysaccharide-encapsulated bacterium, responsible for more than 50% of the invasive disease cases worldwide. Despite the use of available pneumococcal vaccines, the incidence remains high due to the lack of wide serotypes coverage by the current vaccines, which are also expensive and complex to produce since the pathogenic strains for each serotype should be cultivated at a high scale and used for polysaccharide purification [49].

In an effort to establish an approach that avoids the pathogen cultivation through the use of an innovative synthesis platform, Smith et al. [50] succeeded in the production of immunoprotective bacterial polysaccharides (the serotype 3 capsular polysaccharide of *S. pneumoniae*) in the plant cell. The Pneumococcal Type 3 Capsule Synthase Gene (Cps3S) was expressed in *Nicotiana tabacum* following a nuclear stable transformation approach. The coding sequence of Cps3S was fused to the PR1b signal sequence to direct the secretion of the enzyme to the apoplast. The production of the type 3 polysaccharide in the tobacco leaf was proven by saccharide analysis. To test the immunogenicity of the type 3 polysaccharide, mice were intraperitoneally immunized with three doses of apoplast extracts from transgenic or wildtype plants along with alum as adjuvant. Mice were intranasally challenged with the serotype 3 *S. pneumoniae* strain HB565 230 days after the final immunization. Only mice immunized with the transgenic plant extract survived. Thus, the polysaccharide production in plants was demonstrated for the first time. This study opens the path for the implementation of robust platforms for an easy and low-cost production of polysaccharide-based vaccines and even the production of conjugate vaccines in plants since, in theory, the glycosylation machinery of the

plant could transfer heterologous polysaccharides to specific co-expressed recombinant proteins.

3.4. *Bacillus anthracis*

Anthrax is caused by *Bacillus anthracis*, a gram-positive microorganism that produces spores able to remain viable in the environment for centuries in extreme conditions. Once in the host, the spores generate vegetative cells that produce the tripartite complex of anthrax toxins [51]. The inhalational exposure to *B. anthracis* leads to severe respiratory distress causing shock, cyanosis, and death [52]. Given the transmission mode and mortality, anthrax has acquired importance in bioterrorism; thus, the urgent need for rapid response agents to prevent or treat the disease in large populations [53]. The main virulence factor of *B. anthracis* is a multicomponent toxin, the lethal toxin (LeTx), that comprises the protective antigen (PA) and the lethal factor (LF). The PA antigen was expressed in tobacco chloroplasts at yields up to 2.5 mg/g of fresh leaf tissue. The vaccine showed to be stable for several months in stored leaves or crude extracts. In terms of functionality, the chloroplast-derived PA was able, in conjunction with the lethal factor (LF), to be internalized and caused lysis of mouse macrophages *in vitro*. The ORF2 chaperonin from *Bacillus thuringiensis* was also co-expressed but the PA accumulation did not increase [54].

In a further study, the immunogenicity of the plant-made PA antigen was assessed in mice injected s.c. with 5 µg of the partially purified antigen along with Alhydrogel as adjuvant on days 0, 14, 28, and 140. The plant-made PA induced similar IgG titers to those attained with the *B. anthracis*-made fully purified PA (1:300,000). In addition, a group immunized with transgenic plant crude extracts showed lower IgG titers (1:40,000–1:80,000). The activity of the IgG antibodies was assessed in terms of the ability to neutralize LeTx, which is estimated by a decrease in the death of macrophages when exposed to LeTx. It was observed that the immunization with PA-containing crude plant extracts induced strong neutralization titers (1:500–1:7500). In general, a good correlation between the anti-PA antibody levels and neutralization titers was observed. Moreover, the immunized mice were challenged with 1.5 $\times$ 100% lethal dose (LD100) of LeTx, observing that the group immunized with plant-made or *B. anthracis*-made PA and adjuvant survived to the challenge. The immunization with crude extracts presented a survival rate of 80%. The absence of adjuvant in the formulation failed to induce protective immunity [55]. A further study aimed at producing a non-glycosylated form of PA in *N. benthamiana* by co-expressing the peptide-N-glycosidase F (PNGase F) from *Flavobacterium meningosepticum*, which induces an *in vivo* deglycosylation process. The deglycosylated PA possesses a higher immunogenic activity in mice in terms of induction of toxin-neutralizing antibody responses and represents a potential candidate for continuing the development of plant-made vaccines against anthrax [56].

The plant-made PA antigen from *B. anthracis* has also been expressed in plants following a transient expression in *N. benthamiana* with subsequent isolation of the plant-made antigen. In mice, an immunogenicity test using Alhydrogel®

as adjuvant and the i.m. administration was conducted with doses of 45, 15, and 3 µg. Two immunizations with 45 or 15 µg of plant-made PA plus Alhydrogel elicited high titers of anti-PA IgG at day 42, which were maintained at least until day 70. Remarkably, the plant-made recombinant plant-made PA along with Alhydrogel induced anthrax LeTx neutralizing antibodies. In terms of stability, the vaccine was highly stable since after 2-week storage at 4°C on Alhydrogel, the detection of anti-PA IgG and neutralizing antibodies was positive in mice receiving two 7.5-µg doses of adjuvanted plant-made PA. In rabbits, a scheme comprising three immunizations of varying doses of plant-made PA (135, 67.5, 22.5, or 7.5 µg) plus Alhydrogel on days 0, 21, and 42 was implemented. On day 35, strong IgG and neutralizing antibodies were induced after two administrations of the plant-made PA vaccine plus Alhydrogel at 125- and 67.5-µg doses with weaker responses for the 22.5- and 7.5-µg doses. However, after an additional boost (dose 3), all rabbit groups showed strong serum anti-PA IgG/neutralizing antibody responses. Moreover, all rabbit groups subjected to three immunizations were subsequently challenged with a 368 lethal dose 50 (LD50) of aerosolized *B. anthracis* Ames strain spores, observing full protection in all the vaccinated groups whereas unvaccinated animals died 4 days post-challenge [57].

In an effort to produce a vaccine based in minimum protective antigenic sequences, Chichester et al. [58] expressed the domain 4 from PA (PAD4) and domain 1 from the lethal factor (LFD1) fused to a lichenase (LicKM). The chimeric proteins were transiently expressed in *N. benthamiana*. The antigenic activity of PAD4 and LFD1 was retained as revealed by immunoblot analysis. An immunogenicity test was performed in mice groups receiving three 100-µg antigen doses i.p. at 2-week intervals. Complete Freund's adjuvant was used for priming whereas incomplete Freund's adjuvant was used for boosting. IgG responses against PA and LF were induced (antibody titers of 10^6). The plant-made vaccine showed the same immune polarization as the full-length antigens (predominant IgG1, Th2 responses). Remarkably, an *in vitro* neutralization assay with LicKM-PAD4 and LicKM-LFD1 was performed using the line RAW264.7 of mouse macrophage cells, observing complete protection in the samples from mice immunized with LicKM-PAD4 plus LicKM-LFD1.

In a VLP-based approach, the PA-D4 (domain-4 epitope of the anthrax protective antigen) was expressed as a fusion protein along with the hepatitis B core antigen (HBcAg) using a flexible glycine-serine-rich linker to facilitate an appropriate epitope display and folding. Standard nuclear transformation was conducted in low-alkaloid tobacco using the Rubisco promoter to drive the expression and a C-terminal retention signal to target the protein into the ER. The c-myc tag allowed for the purification of the antigen. The plant-made HB/PA-D4 protein was successfully expressed and purified at yields of 1 mg per kg of fresh plant biomass. Moreover, BALB/c mice were immunized i.m. with three doses (3 µg of the soluble plant-derived HB/PA-D4 protein) administered at 2-week intervals. A CpG-oligodeoxynucleotide and alum Alhydrogel were included in the formulation as adjuvants. Although no evidence on the assembly of VLPs was found,

the plant-derived HB/PA-D4 induced humoral responses against both components after the second and third immunizations with high proportion of the IgG2a subclass suggesting a predominant Th1-cellular response [59].

The PA was also expressed in Indian mustard and tobacco chloroplasts with yields of 0.3–0.8% of SP and 2.5–4% of PA, respectively. A macrophage lysis assay was performed using the mouse J774A.1 cell line revealing lysis rates of 23–81% and 80–97% for transgenic mustard samples and transplastomic plants, respectively. In mice, the immunization with *B. juncea* expressing PA plus adjuvant elicited anti-PA IgG responses after i.p. and oral administration. Interestingly, the serum from various immunized groups protected macrophages against anthrax lethal toxin *in vitro*. Moreover, the mice immunized with the plant-made antigens showed an 80% survival rate following a challenge with Sterne strain vegetative cells, whereas orally immunized mice showed a 60% survival rate with no requirement of cholera toxin (CT) as adjuvant to achieve the protection [60].

Another explored approach in plant systems consisted in using a fusion protein called pATR-Fc, comprising a domain from the anthrax toxin receptor (ATR) and the human immunoglobulin Fc fragment using a flexible hinge in between. Nuclear stable expression was implemented in tobacco plants where the Rubisco promoter drove the expression and an apoplastic (Apo) signal, with or without the ER retention signal KDEL, was also fused. Purification of the chimeric antigen was performed by Protein-A chromatography, obtaining fractions with purity above 90%. The yields of the recombinant pATR-Fc protein were up to 3 mg per kg of raw material. The affinity of the plant-made ATR-Fc to PA was comparable to that of the anti-PA mAbs. Moreover, neutralization of anthrax toxin was shown in the pATR-Fc preparation in a dose-dependent manner with an IC50 of 0.6 µg/mL. The plant-made biopharmaceutical was also able to decrease J774A1 macrophage death after a challenge with the anthrax toxin [61].

Tobacco plants stably transformed to express the PA antigen were developed by Aziz et al. [62]. Evaluations conducted in the RAW 264.7 cell line revealed that the soluble protein extracts from different plants added along with LF were able to lyse macrophage cells and showed a biological activity comparable to the native PA (activity in the range of 26–98%).

The domain IV itself from *Bacillus anthracis* protective antigen gene (PAD4) was expressed in tobacco following nuclear and chloroplast expression approaches. The yields were 5.3% and 0.8% of TSP for the chloroplast and nuclear approaches, respectively. An immunogenicity test was conducted in mice following i.p. and oral immunization with or without adjuvant on days 0, 7, 14, 21, and 28. For i.p. immunized groups, the priming was achieved with 5 µg of PAD4 from nuclear transgenic plants using Alhydrogel (20% v/v) as adjuvant. For oral immunization, 10 µg of rPAD4 were coadministered with 5 µg of CT as adjuvant. The plant-made PA elicited antibody titers $>10^4$ upon i.p. and oral immunizations. Predominant IgG1 levels were observed indicating a Th2-polarized response. Significant sIgA levels were detected in feces from orally immunized mice with CT as adjuvant. The antibodies from various groups were efficient in neutralizing the lethal toxin

in vitro. Moreover, the immunized mice were challenged with *B. anthracis*, observing that mice immunized with adjuvanted plant PAD4 had 60% and 40% protection rates, whereas the *E. coli*-derived PAD4 conferred protection at 100% and 80% rates upon i.p. and oral immunizations, respectively [63].

3.5. *Mycobacterium tuberculosis*

The pioneering studies on plant-made vaccines targeting tuberculosis consisted in *Arabidopsis thaliana* plants transformed at the nuclear level to express a fusion protein comprising the B subunit of the *Escherichia coli* heat-labile enterotoxin, as mucosal adjuvant and carrier, and the ESAT-6 antigen [64,65]. Evidence on the antigenicity of both components was provided by ELISA data. The immunogenic potential of this plant-made antigen was evaluated in mice immunized by the oral route observing the induction of T helper responses accompanied of IFN-gamma production. Th2-polarized immune responses were also evident in the Peyer's patch [66].

Further studies were focused on the expression of the antigens Ag85B, MPT83, MPT64, and ESAT6 in potato plants in a tuber-specific manner mediated by the class I patatin promoter. Proteins located at the ER by using the retention signal KDEL. An immunogenicity assessment in C57BL/6 mice immunized by the oral route revealed that cellular responses were induced, which were detected in IFN-gamma and IL-2 production assays in splenocytes as well as expansion of CD4+ and CD8 + T cells. Humoral responses (IgA and IgG) were also induced by this plant-made vaccine. The authors concluded that the potato-made vaccine predominantly induced Th1 responses [67]. Since potato is not proper for oral delivery of raw plant material due to the presence of certain metabolites, further studies in edible crops are the ideal perspective for this vaccine [68]. The expression of ESAT6 and CFP10 antigens was accomplished in carrot roots with yields of 0.056% and 0.002% of TSP, respectively. Interestingly, the authors provided evidence on the induction of cell-mediated and humoral responses following oral immunization in mice [69].

Another research group reported the expression in tobacco plants of the Ag85B and ESAT-6 antigens fused to ELP (TBAg-ELP) [70]. This technology known as ELPylation consists in fusing the protein of interest with the elastin-like peptide (ELP) leading to an increase in protein yields facilitating at the same time protein isolation through the highly efficient, cost-effective approach named inverse transition cycling method [71]. The yields for the production of the tobacco-made TBAg-ELP were 4% of TSP. Following protein isolation, an immunogenicity assessment in C57BL/6 mice was conducted immunizing three times biweekly by the s.c. route using TBAg-ELP along with DDA (dimethyldioctadecylammonium bromide) or MPL (monophosphoryl lipid A) as adjuvants. A positive induction of both humoral and T cell immune responses was observed. Moreover, piglets immunized with crude tobacco leaf extracts by the intradermal route developed humoral immune responses [70].

Other efforts have been focused in the modality of recombinant immune complexes (RICs), which consists of self-polymerized chimeric proteins formed by the antigen of interest

fused to the heavy chain of a monoclonal antibody targeting the antigen of interest. The early secreted Ag85B and the latency-associated Acr antigens were expressed as RICs by fusion with an anti-Acr antibody. Tobacco plants produced the RICs at levels up to 0.2% of TSP. Pure plant-made TB-RICs were able to bind C1q, a complement component, and react on the surface of antigen-presenting cells. The immunogenic potential of this candidate vaccine was assessed in a prime-boost immunization scheme where the test mice were immunized with BCG and subsequently boosted twice intranasally with the TB-RICs. Serum IgG antibody responses were detected with end point titers of 1:8100 (Acr) and 1:24,300 (Ag85B). In addition, positive T cell responses were evidenced in proliferation assays upon Ag85B stimuli. IFN- γ production was also reported when either the Ag85B or Acr stimulus was applied. Remarkably, a significant reduction on lung *M. tuberculosis* infection was observed in BCG-immunized mice boosted with TB-RICs; with lower levels than those observed in the group immunized only with BCG [72].

The use of viral vectors has also been applied with interesting outcomes. The ESAT-6 protein was produced in *N. tabacum* through a PVX vector in which the antigen was fused to the amino terminal of the PVX CP. The 2A peptide from the Foot and Mouth Disease Virus was placed in between to induce a translational skip mechanism and thus either CP and ESAT-6, individually, or the fusion ESAT-2A-CP were produced. Yields in terms of ESAT-6 were in the range of 0.5–1% of TSP [73]. Moreover, a vector derived from Tobacco mosaic virus was devised to produce in *N. benthamiana* plants the antigens Ag85B, ESAT6, as well as a fusion ESAT6:Ag85B. The genes coding for the antigens of interest replaced the CP genes. Agroinfiltration allowed for the expression of the Ag85B antigen at levels of 800 mg/kg fresh weight leaves [74]. With the goal to produce highly immunogenic molecules at the mucosal level, the antigens ESAT-6 (6 kDa early secretory antigenic target) and Mtb72F (a fusion polypeptide from two TB antigens, Mtb32 and Mtb39) were fused to the carrier proteins CT B-subunit (CTB) or LipY (a cell wall protein). The CTB-ESAT6 and CTB-Mtb72F antigens were accumulated in tobacco leaves at levels up to 7.5% and 1.2% of TSP, respectively; whereas the CTB-ESAT6 antigen accumulated in lettuce plants at levels up to 0.75% of TSP. The evaluation of the plant-made CTB-ESAT6 in a hemolysis assay revealed partial hemolysis of red blood cells indicating a functional activity [75].

3.6. Asthma

Asthma is a chronic airway inflammatory disorder, with factors for its development such as genetic predisposition and environmental exposure to inhaled substances and particles. Although asthma is underdiagnosed and undertreated, it is estimated that 235 million of people currently suffer from asthma [76] with children as the most affected group [77]. The conventional management of asthma controls the disease without achieving the cure. Immunotherapies are a viable strategy to alleviate this pathology, with tolerogenic vaccines against asthma under intensive research. Allergen-specific immunotherapy (ASIT) is intended to induce clinical and

immunological tolerance leading to a long-term clinical benefit and associated immunological parameters [78].

In 2003 Smart et al. [79] 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 affirmed that plants can induce allergy, to elucidate this point, it was proven that the systematic consumption of SSA-lupin does not sensitize animals and predispose to hypersensitivity responses. 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 PBS. The purified SSA induces experimental asthma and promotes an inflammatory response comparable to that of the established disease model Ag OVA. The 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) was achieved in the 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. Der p 1 was produced in transgenic rice seeds, consisting of the Der p residues 45–145 that contain 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 hyperresponsiveness (BHR), inhibition of allergen-specific 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 [80].

More recently, in 2013 Li et al. [81] used *N. benthamiana* plants to transiently express tR8, a His-tagged protein derived from the major allergen group 1 of house dust mites (*D. farinae* and *D. pteronyssinus* species). The purified tR8 was obtained at yields of 4.54 ± 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. farinae*-allergic patients. BALB/c mice were intraperitoneally injected with three doses of 10 μ g of relevant allergen and 2% Al(OH)₃ as adjuvant. After the third dose, the mice were challenged by nebulized inhalation of an allergen suspension (0.5 μ g/mL) for 30 min during 7 successive days. The mice were also subjected daily to ASIT by i.p. injection of rProDer f 1, tProDer f 1, and tR8 (100 μ g/mL).

ASIT 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 elaboration of cytokines such as IL-4, IL-13, and IL-5 contribute to the induction of allergy and asthma [82–84]; thus, immunotherapies modifying the Th1/Th2 imbalance are considered promising for asthma.

The effects of tR8 in a mice model of asthma showed 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 of Th1- and Treg-cell differentiation. The IgG1 levels were also significantly downregulated in the tR8 group but IgG2a levels were strikingly raised when compared with other experimental groups. These findings suggest that tR8 can competently rectify the Th1/Th2 imbalance [81].

4. Expert commentary

4.1. Immunization routes to target respiratory pathologies

Plant-made pharmaceuticals offer a promising alternative for medicine since they can be produced in a convenient manner with hosts of easy cultivation, lacking of mammalian pathogens that possess the machinery to perform eukaryotic posttranslational protein modifications. Since the invention of the molecular farming concept more than two decades ago, plant-based vaccines are subject of intensive research and some of the limitations in this field have been successfully addressed. Strategies to enhance antigen expression through innovative approaches achieving proper dosage, using freeze-drying processes, are now available [19]. The immunogenicity of most of the vaccine candidates has been evaluated following parenteral administration. Realistically, short-term advances are expected in this direction. It should be noted that the use of parenterally administered plant-made antigens in prime-boost protocols implies that the antigen must be purified from the plant biomass to yield proper parenteral formulations. However, despite the requirement of costly chromatographic procedures such as IMAC, ionic exchange, and size exclusion procedures, the cost of the raw biomass and its safety in terms of the absence of human pathogens still represent substantial advantages making the plant-based technology very convenient. Nevertheless, the oral delivery of vaccines possesses substantial advantages over the other routes in terms of administration easiness (no trained personnel is required), absence of pain, and low cost. In parallel, it is of remarkable interest that oral immunization can elicit effector immune responses at the respiratory tract mucosa [85]. Therefore, oral plant-based vaccines could be used to induce protective immunity against respiratory conditions. This concept has been proven in preclinical studies using infection models of *Mycobacterium tuberculosis*, *Streptococcus pneumoniae*, *Pseudomonas aeruginosa*, and *Influenza virus*. The observed immunoprotective effects have predominantly correlated with local humoral responses (IgG and IgA) at the respiratory tract [86–90]. Some of the studies have also

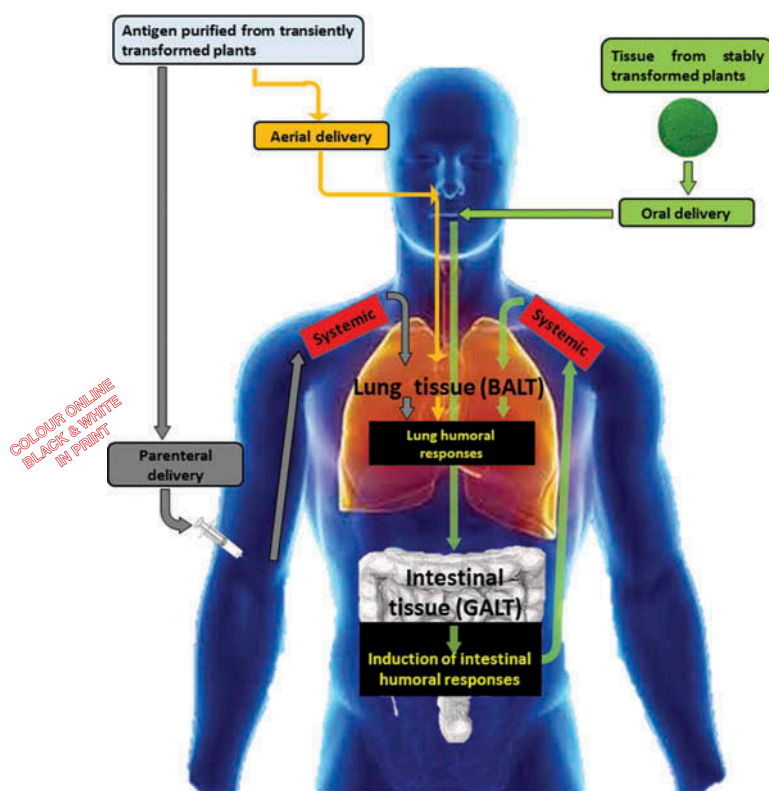


Figure 1. Potential uses of plant-based vaccines in the fight against respiratory diseases in humans. Plant-based platforms, especially those based in transient expression can reach high yields that allow for antigen purification and its use for immunization by airway and parenteral routes. On the other hand, oral vaccines can be formulated with nuclear-stably transformed plants or transplasmatic plants. Oral immunization can support the expansion of B and T cells at the gut associated lymphoid tissues (GALT) that can subsequently migrate to the lung mucosa and mediate cellular and humoral effector mechanisms, thus immunization by the oral route can be performed as a boosting strategy. The parenteral immunization may result in the induction of robust systemic and lung mucosa responses and can be performed to prime the immune system. Airway immunization is proposed as a straight forward approach for the induction of immunity at the lung mucosa through stimulation of the elements present in the Bronchus-Associated Lymphoid Tissue (BALT).

shown that a single oral immunization leads to modest responses when compared to the parentally administered vaccines; however, subcutaneous priming followed by oral boosting led to strong immune responses that are equal or superior in potency to those elicited by parenteral, conventional immunization schemes [86,91,92]. Therefore, oral plant-based vaccines could be applied as low-cost boosts in schemes comprising parenteral priming. These possibilities are illustrated and summarized in Figure 1. One important aspect to consider is the tolerogenic nature of the gut-associated lymphoid tissues, which can be overridden by the use of adequate adjuvants and optimized immunization schemes [93].

In addition, it should be considered that the transient expression systems in plants also allow for the production of parenteral formulations, especially when transient vectors of high productivity are used. Since airway immunization also has shown a promising potential to induce strong cellular responses, immunization by this route using purified plant-made antigens is proposed as a relevant research path [94,95]. Although immunizations via the i.t. or i.n. routes have been

narrowly studied for plant-made antigens, these approaches are considered to have an important potential and conducting more efforts in this direction will be of relevance for the field in terms of using more accessible immunization routes with the possibility of achieving attractive immunological profiles.

Respiratory diseases face differential challenges in terms of the development of effective vaccines. For instance, the development of plant-based vaccines against RSV seems to be a long-term goal since the correlates of protection and safety are still a concern even in conventional vaccines. Thus, fundamental research on immunization is needed [96]. Today, the infection mechanism of RSV is well characterized and this could be beneficial to the development of a potent and efficient vaccine against RSV. Future assessments with new vaccines should look for an animal model exhibiting similar responses to those occurring in humans; however, none of the models reproduces RSV infection at a high degree. At the present time, cotton rats are the most adequate animal model for RSV infection since they are more susceptible to RSV than mice and also exhibit more prominent neutrophil infiltration [97]. Therefore, substantial efforts are still required to overcome the obstacles to achieve effective and safe RSV vaccines. The challenging goals comprise avoiding the RSV disease enhancement seen with inactivated vaccines and using formulations of needle-free administration able to induce local and systemic immunity with no accessory adjuvants. The developments for *S. pneumoniae* are still in their infancy but the expression of polysaccharides represents a novel trend in the field with promising prospects [98]. Research in this direction will be critical.

Different plants have been used for the production of candidate tolerogenic vaccines against asthma, which is beneficial since the incidence of allergic diseases seems to be rising. The efforts to produce a successful tolerogenic vaccine against asthma have to include nonresponsiveness, symptom release, immune deviation of the Th1/Th2 imbalance, and/or induction of allergen-specific antibodies to prevent immediate-type reactions [99]. The promising potential of asthma plant-based tolerogenic vaccines identified by the rectification of the Th1/Th2 immune balance indicates a prospect for such developments. The high number of candidates targeting tuberculosis and anthrax indicates a great potential to achieve plant-based vaccines in an advanced degree of development in the midterm. Importantly, the case of influenza vaccines is the most noticeable example since the candidates are under clinical evaluation. The development of new products will be accelerated by the investment of companies along with cooperation with nongovernmental organizations concerned with vaccine manufacture, developing country-scientists, and institutions for both research and development.

It is considered that innovative antigen designs could also add key advances in this field. Remarkably, some multiepitope or multicomponent vaccines have been produced in plant systems with a promising potential for the induction of broad immune responses with the goal to protect against several viral variants [100,101]. This aspect has been neglected for the case of respiratory diseases and constitutes a relevant research path, especially for highly variable virus, where

repeated vaccinations are performed to appropriately protect against the new circulating strains.

4.2. Production and regulatory aspects

In terms of commercial production and regulatory issues, important advances were achieved during the last years. Influenza plant-made vaccines parentally administered will be perhaps the first to be commercialized due to the assumed success of phase III clinical trials. These candidates are produced using transient expression systems based on viral vectors. In fact, an automated platform for exploiting this technology under a cGMP process has been established [10]. However, it is important to recognize that even though it was assumed that a plant-produced vaccine could be available between 1 and 3 months after the pandemic virus emerges, the potential regulatory delays associated with vaccine approval have not been considered, which would impact the actual release of the vaccine to the public [102].

This focus, however, is not ideal in terms of production since purification is still required implying higher costs. The use of viral vector-mediated expression systems in edible crops, in a way lacking of agroinfiltration, could constitute a viable system free of bacterial contaminants that may allow for oral vaccine formulation using freeze-dried plant biomass, therefore, avoiding the need for purification. In the case of transplastomic technologies, although no complex enveloped VLPs can be produced, epitope-based vaccines such as those extensively studied and based in the mucosal adjuvants CT B subunit and heat labile toxin B subunit could be produced under this strategy [64]. In terms of scale-up and production under GMPs, a recent report by Su et al. [3] is of interest since the potential of stably chloroplast-transformed lettuce grown under hydroponic conditions in a cGMP process can be effectively processed to obtain a proper pharmaceutical form (capsules containing freeze-dried leaves). This approach indicates that the production of plant-based vaccines at a larger scale, with lower costs, and under GMPs is viable [3].

In terms of dealing with regulatory agencies to approve plant-made biopharmaceuticals for human use, the cases of a couple of biopharmaceuticals that have gone through regulatory agencies constitute a valuable reference in this topic. These comprise a carrot-made glucocerebrosidase approved for human use as treatment of Gaucher's disease [103] and a monoclonal antibody targeting HIV, whose regulatory approval for the GMP-production and evaluation in phase I clinical trial were accomplished [104]. These strides are of key importance to mature the regulatory framework for plant-made biopharmaceuticals looking to smooth the path for the approval of the upcoming plant-made vaccines [105]. The evaluation of Influenza vaccines in clinical trials represents a key reference in the field that will facilitate the evaluation of other vaccines at the clinical level following the established path for ensuring the compliance of the regulatory agencies [9].

5. Five-year view

It is clear that plant-based vaccines have the potential to positively impact the global-scale immunization targeting

respiratory diseases. A substantial progress is envisioned for the following five years in this field. For instance, the clinical trials conducted thus far to assess influenza vaccines support a realistic expectation to have these vaccines in the market in the short term. The generation of new insights on the immunoprotective potential of plant-based vaccines by conducting immunization protocols comprising priming and boosting by distinct routes is also expected, including airway and oral immunization as either priming or boosting agents. Such immunological aspects will generate critical advances in the field since no well-characterized immunization schemes have been reported for most of the candidates thus far. The regulatory approval of the new vaccines is expected to follow a smoother path due to the fact that a plant-made biopharmaceutical was approved for human use four years ago. New immunogen designs, especially those immunogenic by mucosal routes as well as innovative expression approaches, will also contribute to the expansion of paths in the development of highly effective and low-cost vaccines against respiratory conditions. In conclusion, plant-based vaccines are a promise for the biomedical field and the respiratory diseases, influenza especially, are expected to be the pioneering target resulting in enormous benefits for the global health in the short term.

Key issues

- Respiratory diseases (RD) constitute a threat for global human and animal health.
- In the search of new immunization approaches to fight RD, plants have been used as biofactories of immunotherapeutic agents.
- Plants could also serve as the delivery vehicles for oral vaccines
- Several plant-based vaccines targeting respiratory pathologies are under development and evaluation in preclinical trials
- The case of plant-made influenza vaccines is remarkable since some of the candidates are currently under clinical evaluation
- The main perspectives for the field comprise optimizing and diversifying the immunization schemes as well as achieving the regulatory approval of influenza vaccines

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Declaration of interest

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