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REVIEW

Corn-based vaccines: current status and prospects

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Abstract

Main conclusion **Corn is an attractive host for vaccine production and oral delivery. The present review provides the current outlook and perspectives for this field.**

Among seed-crops, corn represents a key source of biomass for food, fuel production, and other applications. Since the beginning of the development of plant-based vaccines, corn was explored for the production and delivery of vaccines. About a dozen of pathogens have been studied under this technology with distinct degrees of development. A vaccine prototype against enterotoxigenic *Escherichia coli* was evaluated in a phase I clinical trial and several candidates targeting bacterial and viral diseases are under preclinical evaluation. The present review provides an updated outlook on this topic highlighting the employed expression strategies; perspectives for the field are also provided.

Keywords *Zea mays* · Maize · Seed · Vaccine · Molecular farming · Oral immunization · Gamma zein promoter · Vacuole targeting

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Introduction

The technology of plant-based vaccines has been explored for more than two decades and several proofs of concept have been generated. Along this path, several maturation steps have been achieved for the technology, which are reviewed elsewhere (Govea-Alonso et al. 2014). For instance yields improvement and increased biosafety, as well as stability of the vaccine formulations, are key aspects addressed through several approaches (Lakshmi et al. 2013). Currently, the most advanced plant-based vaccines rely on platforms based on transplastomic technologies and transient expression systems mediated by viral elements (Gleba et al. 2007; Daniell et al. 2016). The former offers relatively high yields and enhanced biosafety since the transgene is maternally inherited and thus not transmitted by pollen, while the latter offers high yields and short production time. For both platforms, GMP-compliant processes have been developed at an industrial scale to produce vaccines against influenza and tolerogenic therapies that prevent the induction of blocking antibodies in patients with hemophilia B (D'Aoust et al. 2010; Su et al. 2015). Another possible approach offering substantial advantages, such as full containment and optimization of growth conditions, is the bioreactor-based plant biomass production (Huang and McDonald 2012).

Another key improvement was changing the concept of 'eating transgenic fruits as vaccine' to the concept of using processed plant material instead, which offers enhanced stability and more accurate dosage (Pniewski et al. 2011). A particular expression modality is focused on targeting seed crops, which offers the following attractive advantages: (1) Provided that seeds are natural reservoirs of several nutrients, they possess high

biosynthetic capacity and thus can produce valuable recombinant proteins at convenient yields (Boothe et al. 1997; Karg and Kallio 2009). For instance in the case of maize a period of 3–4 months is required to yield several thousands of seeds per generation and recombinant protein production can be scaled-up to several hundreds of grams (in a range of 0.2–2 kg) per acre (Hood et al. 1999; Tacket et al. 2004). (2) Optimized genetic engineering technologies are available for the most important seed crops. (3) Seeds offer a very stable matrix for the preservation of recombinant proteins at room temperature (Stoger et al. 2005). Several compartmentalization alternatives can be focused in the seed cells to favor protein stability and other variables such as antigen release or accumulation levels, which include targeting protein bodies of the endosperm (escaping proteolysis during maturation in the cytosol and during programmed cell death in the final stages of cereal grain maturation) (Wu et al. 2007; Yang et al. 2012), protein storage vacuoles (Lau et al. 2010), and oil bodies (Deckers et al. 2014). (4) Given that several seeds are edible, they can serve as a convenient source of biomass for the formulation of oral vaccines following a simple processing. Since seeds are highly stable, the formulation can be performed after a period of storage without altering the antigen content, in contrast with leaf tissues where a limited shelf life exists requiring immediate processing after harvesting (Fischer et al. 2004). Another key aspect is the reduction of antigen degradation since the plant biomass derived from seeds can delay proteolysis, improving bioavailability of the immunogen to the gut associated lymphoid tissues (Rosales-Mendoza and Salazar-González 2014). (5) Packaging, storage, processing, and distribution technologies are well developed and established for most seed crops, thus facilitating formulation, dosage, and delivery of subunit oral vaccines (Garg et al. 2007; Moravec et al. 2007). When required, protein purification could be also facilitated in seed-based systems due to low content of phenolic compounds and pigments with lower complex mixtures of proteins and lipids when compared to those of leaves (Daniell et al. 2001; Tiwari et al. 2009). The above-mentioned characteristics of seeds clearly support their use as low-cost and convenient platforms for the production of subunit vaccines.

To date, the expression of several subunit vaccine antigens (Orellana-Escobedo et al. 2014) and therapeutic antibodies (Stoger et al. 2000) in plant seeds has been achieved. The present review is focused on the experiences gained by several research groups on the use of corn as an attractive vaccine expression and delivery system (Fig. 1; Table 1).

Corn-based vaccine prototypes

Vaccines targeting bacterial toxin subunits to prevent enteric infections and unrelated pathologies

Escherichia coli strains (ETEC) cause diarrhea in humans and animals leading to a significant epidemiologic impact (Kopic and Geibel 2010). The B subunit of the ETEC heat labile enterotoxin (LTB) is an attractive antigen for vaccine development against this pathogen since it induces toxin neutralizing antibodies and in fact may cross-protect against the cholera toxin (CT) (O'Ryan et al. 2015). Several studies on the production and characterization of LTB in maize have been reported. Maize seeds expressing LTB are proposed as a plant-derived vaccine for traveler's diarrhea and as an adjuvant for co-administered proteins (Karaman et al. 2006). Chikwamba et al. (2002a) produced a functional LTB in maize. A synthetic LTB gene was under control of the γ-zein promoter, the translational enhancer leader sequence from the Tobacco Etch Virus (TEV), and the soybean vegetative storage protein terminator (vsp). Plants were transformed using microprojectile bombardment. The recombinant antigen was analyzed and quantified by western blot and ELISA, respectively. LTB expression reached up to 0.05% of total soluble protein (TSP). The preparation of pellets consisted of maize powder mixed with sterile distilled water and molded into a cylinder. The mice bioassay consisted of 4 oral immunizations with a pellet containing 10 μg LTB on days 0, 3, 7, and 21. The mice were then challenged with 25 or 50 μg of CT or LT toxins. The results showed elevated IgG and IgA serum antibodies and elevated fecal IgA against the diarrhea-inducing toxins CT and LT. Moreover, mice fed with transgenic material showed reduced gut/carcass ratios indicating that the maize-synthesized LTB has biological and immunological functions comparable to the native LTB (Chikwamba et al. 2002a).

After their previous work expressing LTB, Chikwamba et al. (2002b) examined the role of promoters and the SEKDEL retention motif in LTB accumulation in callus and kernels. The 35S CaMV and the γ-zein promoters were used for the expression of LTB with or without the SEKDEL endoplasmic reticulum-retention sequence. The tobacco etch virus translational enhancer leader sequence was included in all the constructs as well as the soybean vegetative storage promoter terminator. Maize plants were transformed by microparticle bombardment. The quantification of LTB in maize revealed a production up to 0.04% of total aqueous-extractable protein (TAEP) in callus and 0.01% TAEP in R_1 kernels using the constitutive promoter. The SEKDEL motif enhanced recombinant production in

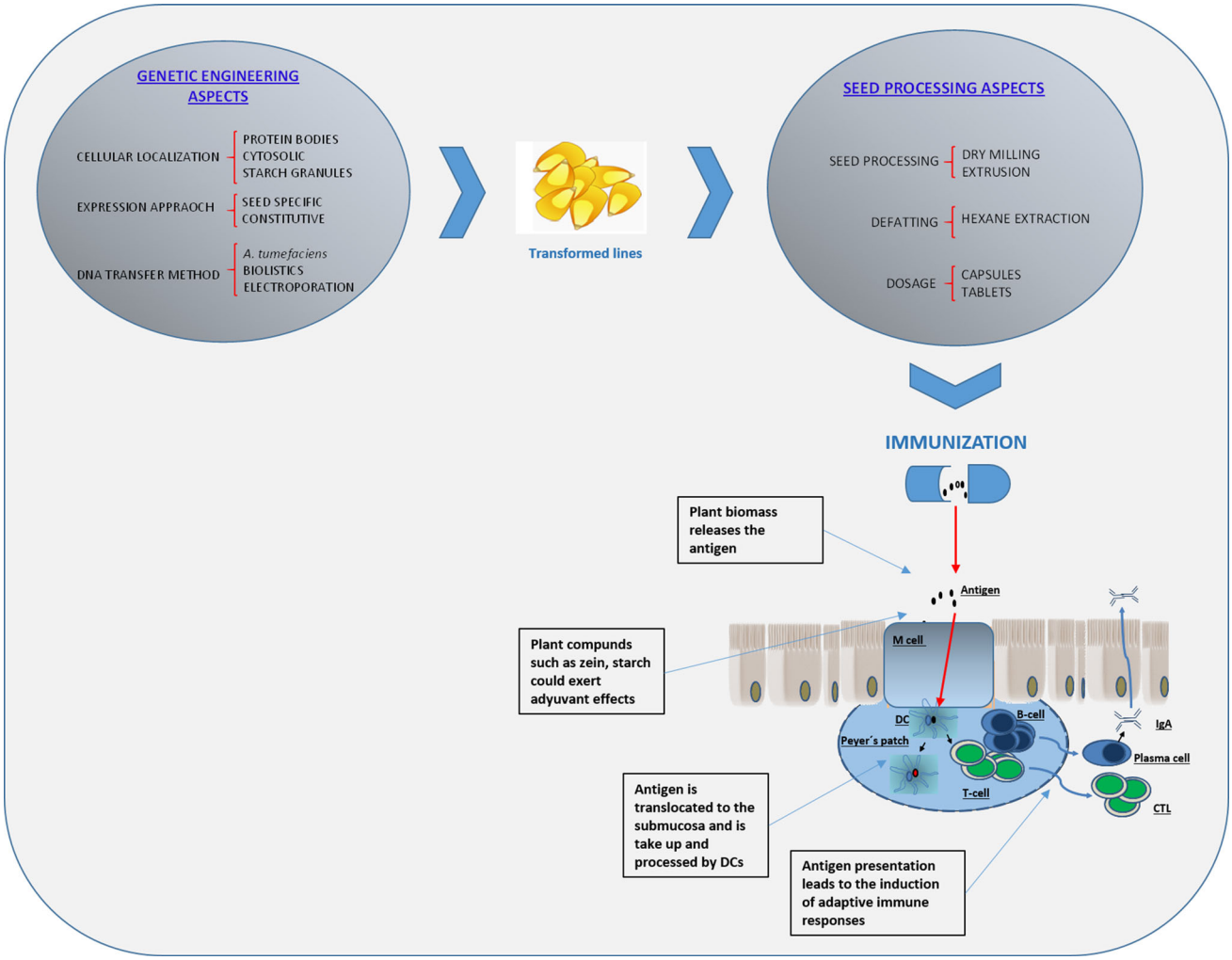

Fig. 1 Plant biotechnology approaches for the design of oral corn-made vaccines. The current strategies for the accumulation enhancement of recombinant antigens in maize include antigen localization in specific organelles (e.g. ER, vacuole, protein bodies) and the use of seed-specific promoters. After selection of transgenic lines based in transgene detection and antigen quantification, the processing of plant material may comprise chemical processes that allow concentrating the antigen. Oral vaccines can be formulated in a straightforward manner using milled corn material. Several approaches can be followed to favor the induction of effective adaptive immune responses through the intestinal mucosa including the use of immunogenic/adjuvant carriers and antigens in the form of virus-like particles. In addition endogenous compounds from the corn material such as zein, starch, and lipids, as well as the bioencapsulation effect exerted by the plant cell may enhance the vaccine activity

combination with the gamma zein promoter. Up to 0.07% TAEP was reached using the gamma zein promoter in R_1 kernels. A maximum of 0.3% TAEP was measured in R_3 seeds using the constitutive promoter and up to 3.7% TAEP in R_3 seeds for the transgenic lines carrying the γ-zein promoter (Chikwamba et al. 2002b).

Later, Chikwamba et al. (2003) produced LTB in starch granules observing that the accumulation levels reached up to 1.3 µg/g (dry weight, DW) of starch fraction and 2 µg/g DW of endosperm. An advantage in producing protein using granules is the protection from degradation by proteases upon oral administration. In this study, the LTB produced in starch granules was more resistant to peptic cleavage in simulated gastric fluid (SGF) digests. Additional advantages of such approach comprise the possibility to co-purifying the recombinant protein with starch as well as a high thermostability (Chikwamba et al. 2003).

Thereafter, transgenic maize seeds (R_4 generation) expressing LTB were developed as described by Chikwamba et al. (2002b). Corn pellets (1.87 g each, DW) were made with ground transgenic maize seeds containing 10 µg LTB/pellet. BALB/c mice were fed with corn pellets on days 0, 7, 21, and 35. Specific IgG antibodies were detected at levels significantly higher than those for the control group on day 13 (reaching a peak on day 27) and remained significantly higher throughout the study. Similarly, specific serum IgA was first detected on day 13 reaching a

Table 1 Recent innovative examples of corn-based vaccine prototypes

Antigen (target disease)	Genetic constructs	Immunological evaluation	Findings	References
ApxIIA antigen from *Actinobacillus pleuropneumoniae* (Porcine pleuropneumoniae)	Vector: pMYV (616–623) Promoter: 35s CMV Terminator: T-Nos Transformation method: Agrobacterium Selection approach: paromomycin resistance (*nptII* gene)	Four-week-old ICR female mice were divided into a non-vaccinated control group and groups that received injections (s.c.) of corn-extracts containing the CTB-ApxIIA#5 fusion protein or full-size ApxIIA mixed with complete Freund's adjuvant. Mice were boosted with soluble antigen extracts mixed with incomplete Freund's adjuvant	Immunogenicity was confirmed by the detection of ApxII-specific memory B cells producing IgG and secretory IgA, as well as the higher secretion of IFN-γ and nitric oxide in immunized mice compared with control	Kim et al. (2010), Shin et al. (2011)
M protein from PRRSV (porcine reproductive and respiratory syndrome)	Vector: pAHCM Promoter: ubiquitin Terminator: Nos Transformation method: biolistic Selection approach: Bialaphos resistance (*bar* gene)	Eight-week-old female BALB/c mice were divided into a test group and a control. Each immunized mouse received 4 doses (on days 0, 15, 30, and 45) of 30 mg of transgenic corn powder (2.6 μg of M protein) plus Sigma adjuvant system® by gavage. Samples of feces and blood were collected before the first dose and 2 weeks after each immunization	Serum (IgG) and fecal (IgA) M protein-specific antibodies as well as neutralization antibody titers were increased after each boost. Moreover, production of PRRSV-specific IFN-γ, but not IL-10, in splenocytes from immunized mice was observed	Hu et al. (2012)
Nucleoprotein (NP) from influenza A virus H3N2 (influenza)	Vector: pHN05 Promoter: 27 γ-zein Terminator: VSP Transformation method: Agrobacterium Selection approach: Bialaphos resistance (*bar* gene)	Pig study 7-week-old pigs were randomly assigned into two groups. Immunized animals received two doses (i.m., day 0 and 21) of maize extract (16 μg NP) mixed with adjuvant (Benchmark BioLabs, 4:1). Sera samples were collected at days 0, 21 and 27 post-immunization Mice study Eight-week-old female BALB/c mice received a prime immunization (s.c.) with 20 μg of maize extracts mixed with Imject Alum adjuvant, while boosts on days 21 and 42 were administered orally without adjuvant	Pigs: significant serum NP-specific IgG response was achieved on day 27 (6 days after 2nd boost immunization) Mice: anti-NP IgG, IgG1, and IgG2a levels were increased in orally immunized mice on day 28 and 56. Antigen-specific stimulation of IL-4 secretion was elicited in splenocytes from immunized mice	Nahampun et al. (2015)
G protein (GP) from rabies virus (rabies in sheep)	Vector: pGPTV-bar and pGHCNS Promoter: ubiquitin Terminator: not specified Transformation method: biolistic Selection approach: Basta resistance (*bar* gene)	Three-month-old Pelibuey sheep were grouped: Group 1, sheep fed 0.5 mg of rGP in 20 g of ground maize kernels; Group 2, sheep fed 1.0 mg of rGP in 40 g of ground maize kernels; Group 3, sheep fed 1.5 mg of rGP in 60 g of ground maize kernels; Group 4, sheep fed 2.0 mg of rGP in 80 g of ground maize kernels; Group 5, sheep vaccinated with an inactivated rabies vaccine (i.m.), and Group 6, animals fed 40 g of non-transformed ground maize kernels Sheep were bled at 0, 30, 60, 90 and 120 days after immunization and were challenged on day 120 by the injection of 10^7 sheep ICLD50 of rabies virus CASS-88 strain	IgG titers in Group 4 (2 mg rGP) and the positive group produced the highest serum titers of neutralizing antibodies from day 30 to day 120. Moreover, immunized sheep were protected (survival rate) after challenge by 50% (0.5 or 1.0 mg rGP), 66% (1.5 mg rGP) and 83% (2 mg rGP and commercial vaccine)	Loza-Rubio et al. (2012)

Table 1 continued

Antigen (target disease)	Genetic constructs	Immunological evaluation	Findings	References
Hepatitis B surface antigen (HBsAg) from hepatitis B virus (hepatitis)	Vector: (HBE construct designed) Promoter: Globulin 1 Terminator: PinII Transformation method: Agrobacterium Selection approach: glufosinate resistance (*bar* gene)	BALB/c mice were vaccinated (i.p.) with 0.5 µg Recombivax® (commercial HBsAg vaccine) on day 0 and orally boosted 13, 15, and 17 weeks post-injection. Each boost consisted of three consecutive daily doses of defatted germ (2.5 mg HBsAg per boost) with or without adjuvant (LT). Blood and fecal samples were collected every 2 or 3 weeks	Anti-HBsAg IgA (fecal and serum) and IgG (serum) were strongly increased after each boost immunization with or without adjuvant	Hayden et al. (2012)
Hepatitis B surface antigen (HBsAg) from hepatitis B virus (hepatitis)	Vector: (HBG construct designed) Promoter: Globulin 1 Terminator: PinII Transformation method: Agrobacterium Selection approach: glufosinate resistance (*bar* gene)	BALB/c mice were vaccinated (i.p.) with 0.5 µg Recombivax® with boostings on days 112 and 126. For each boost, two wafers were provided per day for three consecutive days (4 mg of HBsAg per boost). Blood and fecal samples were collected	Fecal IgA anti-HBsAg was strongly induced. Moreover, supercritical fluid extraction (SFE) of HBsAg from maize enhanced high serum IgG and IgA anti-HBsAg titers in orally boosted mice	Hayden et al. (2014)
Hepatitis B surface antigen (HBsAg) from hepatitis B virus (hepatitis)	Vector: (HBG construct designed) Promoter: Globulin 1 Terminator: PinII Transformation method: Agrobacterium Selection approach: glufosinate resistance (*bar* gene)	BALB/c mice were immunized (i.m.) with 0.25 µg Recombivax® and orally boosted with SFE-defatted wafers on week 13, 15, 47, and 50. Doses of 2.4 mg HBsAg were given for the first and second boosts, while doses of 8.9 mg HBsAg were given for the third and fourth boosts). Blood samples were collected every 2–4 weeks until 3 weeks following boost 2, then every 10 weeks until boost 3, and finally every 2–4 weeks until the terminal bleed. Fecal samples were collected twice a week	Mice boosted orally with wafers containing HBsAg showed increases in fecal and serum IgA titers. Long-term memory in the orally-immunized mice was evidenced by sustained fecal IgA, and serum IgA and IgG over one year. Moreover, sharp antibody increases were induced after re-boosting at 47 and 50 weeks post-primary injection	Hayden et al. (2015)

peak on day 27. By contrast, the highest concentration of fecal IgA was recorded on days 27 and 41, which was sustained until day 62. Interestingly in the same report booster memory immune response (recall) was assessed, revealing high levels of specific IgG and IgA in sera and specific IgA in feces of the groups immunized with corn-LTB as early as 7 days after the booster (recall) antigen administration when compared to the control group. In another experiment, aged (14 months old) mice were orally immunized with corn LTB vaccine to compare the immune responses observed in young mice. Specific serum IgG antibodies were observed on day 13 in aged mice but significantly lower than in sera from young mice remaining significantly lower throughout days 20–41. Overall, anti-LTB IgA concentrations in sera from aged mice were significantly higher than those from young mice. On the contrary, no significant age effect on the anti-LTB fecal IgA levels was observed; however, a very rapid antigen-

specific secretory IgA response was shown as early as day 6 in fecal extracts of the aged mice when compared to young mice. This report highlighted that the corn-made LTB vaccine induces long lasting immune responses in mice (Karaman et al. 2006).

Karaman et al. (2012) produced LTB and the CT B subunit (CTB), which is similar in sequence and function to LTB, in the endosperm of maize kernels. The genes were expressed under control of the maize specific γ-zein promoter linked to the translational enhancer of the Tobacco etch virus. Maize immature embryos were used for transformation using microprojectile bombardment. The antigens were detected by Western blot and quantified by ELISA in maize protein extracts; the yield in T_1 generation seeds reached up to 0.0197% TSP. Test mice were orally immunized four times with 5 or 10 μg of maize-made CTB or LTB. High levels of anti-CTB and anti-LTB IgG were observed in groups vaccinated with maize-made antigens. The analyses of fecal material showed production of anti-CTB and anti-LTB IgA in groups vaccinated with maize-derived CTB and LTB. A challenge with CT and LT toxins showed significant protection in mice vaccinated with 5 μg of the maize-derived antigens (Karaman et al. 2012).

Streatfield et al. (2003) expressed LTB targeting different cellular localizations: cell surface (barley α-amylase signal sequence), endoplasmic reticulum (barley α-amylase signal sequence in 5′ end and SEKDEL ER retention signal in 3′ end), plastid (glycogen synthase signal sequence), vacuole (barley aleurain signal sequence), and nucleus (simian virus 40 large T antigen sequence). The highest accumulation of LTB in T_1 seeds was observed in plants transformed with the vacuole targeting signal, where levels of 12% of TSP were reached followed by cell surface, endoplasmic reticulum, nucleus, plastid, and cytoplasm. In the absence of any sub-cellular targeting signal, the antigen levels decreased 20,000-fold in comparison to those for the vacuolar targeted protein (Streatfield et al. 2003).

After generation of transgenic material in 2003 (Streatfield et al. 2003), Tacket et al. (2004) conducted a human trial with maize expressing LTB. The plant material was processed to feed humans by removing fat, oil, and impurities; and grinding to a small particle size followed by moisture adjustment to concentrate the LTB antigen. Patients received 2.1 g (DW) of defatted LTB corn germ meal containing 1 mg of LTB and 2.1 mg of control corn. Additional doses were administered on days 7 and 21. The results showed that the LTB antigen expressed in transgenic corn was well tolerated and immunogenic in humans with increases in serum IgG and IgA, and stool IgA. The highest antibody increases were observed after the second or third dose of the transgenic corn-vaccine. Moreover, cells derived from the intestine secreting LT-specific antibodies could be detected in the peripheral blood (Tacket et al. 2004).

Of interest is the report made by Kosaki et al. (2008) in which the toxicological effects of transgenic corn expressing LTB were evaluated in two soil invertebrates commonly used as acute and chronic toxicity models. The expression of functional pentameric LTB was achieved in kernels using the maize endoplasm-specific γ-zein promoter in accordance with the previous report by Chikwamba et al. (2003). The transformation of maize plants was achieved using the biolistic method. Finely ground dried maize kernels were passed through a 425 μm mesh for a better exposure to the test organisms. The springtail *Folsomia candida* and the earthworm *Eisenia fetida* were exposed during 28-day periods with standard soils mixed with transgenic maize. The expression level of LTB in transgenic maize was up to 30 μg of LTB/g DW of kernel as determined by ELISA. Interestingly, no toxicological effect was observed in the survival and reproduction of *F. candida*; neither the survival nor the growth of *E. fetida* was affected. These results indicated no evidence on LTB-mediated adverse effects on *F. candida* and *E. fetida* suggesting a limited ecological risk. This is an important aspect since LTB from maize is strongly immunogenic in mammals, thus the potential toxicological effect of LTB from maize in wildlife needs to be considered for GMP of plant-based vaccines (Kashima et al. 2016).

Since CTB and LTB can also serve as immunogenic carriers of unrelated antigens, chimeric proteins based on the toxin subunits may constitute bivalent vaccines, a concept that has been explored in maize. *Actinobacillus pleuropneumoniae* is the causative agent of porcine pleuropneumonia, which is worldwide distributed and causes severe economic losses in pig-rearing countries. Kim et al. (2010) expressed the full-length ApxIIA antigen from *A. pleuropneumoniae* as well as the neutralizing epitope of ApxIIA#5 fused to CTB. Corn transformation was achieved through an *Agrobacterium*-mediated method (Kim et al. 2010). Thereafter, Shin et al. (2011) conducted a study in mice where the test animals were subcutaneously injected with corn-derived ApxIIA and CTB-ApxIIA. The mice were subcutaneously boosted with 200 μL of soluble antigen extracts mixed with complete Freund's adjuvant and subsequently boosted twice with the same antigen mixed with incomplete Freund's adjuvant. An increase in serum ApxIIA-IgG antibodies was observed in mice boosted with either ApxIIA or CTB-ApxIIA#5, but no significant differences of anti-ApxIIA IgA between these groups and the control group were observed. Higher production of ApxIIA-specific IgG and IgA memory B cells was observed in spleen cells of mice boosted with CTB-ApxIIA#5. The secretion of IFN-γ (Th1 type cytokine), IL-4 (Th2 type cytokine), and NO from spleen cells was measured. The results showed production of IFN-γ in the group injected with full-length ApxIIA, whereas no

production of IL-4 in any of the groups was detected. NO production was observed in the CTB-ApxIIA- and full-length ApxIIA-treated groups (Shin et al. 2011).

In another report, epitopes from the human peptide 'amyloid beta' (AP_{42}) were fused to LTB generating an orally administered corn-derived vaccine against Alzheimer's disease, which is the leading cause of dementia (Hickman et al. 2016). The LTB-AP_{42} was expressed in embryogenic maize calli under the 35SCaMV promoter using microprojectile bombardment for transformation. The fusion construct was co-bombarded with the selectable marker gene construct that conferred resistance to the herbicide bialaphos by expression of the bar gene. An enzyme-linked immunosorbent assay (ELISA) failed to detect amyloid beta using human amyloid beta (1–42) as standard. In contrast, a western blot analysis revealed a band corresponding to 55 kDa pentameric LTB, indicating the possible cleavage of AP_{42} from the fusion. Although no immunogenicity tests in this work were performed, the potential use of corn as biofactory of vaccines against Alzheimer's disease was demonstrated (Karaman 2006). Remarkably several plant species such as rice, soybean, tomato, and potato have been explored over the last decade as vaccine biofactories and oral delivery vehicles using 'amyloid beta' as antigen (Rosales-Mendoza et al. 2014).

Viral diseases

Hepatitis B

Hepatitis B virus (HBV) causes one of the most common and hazardous viral diseases in humans and low cost HBV vaccine production platforms are needed (Hemming et al. 2016). Hayden et al. (2012) expressed a bioencapsulated hepatitis B surface antigen (HBsAg) at distinct subcellular localizations: (1) to the cell wall using the type B barley alpha amylase signal sequence (BAASS) and the maize polyubiquitin promoter, (2) to the amyloplast using the waxy gene signal peptide and the polyubiquitin promoter, (3) to the endoplasmatic reticulum using KDEL signal and the 1.4 kb globulin 1 promoter along with BAASS. Maize plants were transformed by *Agrobacterium*-mediated method and the transgenic lines were selected by recombinant protein yields. The presence of HBsAg was confirmed by immunoblot and the expression levels were analyzed using ELISA showing that the globulin 1 promoter allowed plants to produce HBsAg up to 0.25% TSP. Further breeding of seeds enhanced the expression of HBsAg to 0.46% TSP (71 μg/g DW). Bulking of all ears of the generated hybrids resulted in material containing 0.20% TSP (29 μg/g DW). BALB/c mice were given a primary intraperitoneal injection of 0.5 μg Recombivax® on day 0 and boosted 13, 15, and 17 weeks post-injection with HBsAg or non-transgenic germ. The boosts consisted of three consecutive daily doses with 5 g of germ (a total of 2.5 mg HBsAg per boost) pelleted with 5 mL of ddH_2O or ddH_2O + 25 μg LT (R192G/L211A) as adjuvant. Serum anti-HBsAg IgG and IgA and IgA from feces were detected using sandwich ELISA. The mice developed a primary antibody response to the Recombivax injection that peaked at week 10 and a second peak in serum IgG and IgA was observed after oral boosting with the HBsAg germ, while a decrease appeared in the control group. IgA in feces showed superior levels (more than 50% increase) in mice fed with transgenic corn. The use of LT as adjuvant did not modify the humoral response against HBsAg (Hayden et al. 2012).

Hayden et al. (2013) produced HBsAg in maize under control of the 1.4 and 4 kb globulin 1 promoters fused to a codon optimized type B barley alpha amylase signal sequence. One of the constructs contained a vacuolar targeting signal derived from barley aleurain and all of them contained a potato protease inhibitor II (PinII) termination sequence. The transformed plants were obtained by *Agrobacterium*-mediated method and single T1 seeds were analyzed by ELISA for HBsAg accumulation. Interestingly, a construct with the 3× globulin 1 promoter reached expression levels of 0.51% TSP. A stability test was carried out with ground maize exposed for one week or one month to a range of temperatures (−20 to 80 °C). Western blot and ELISA analyses were subsequently performed to assess antigen concentrations. The HBsAg levels remained abundant at −20 and 55 °C but were significantly degraded at 80 °C. At −20 °C maize contained approximately 50 μg HBsAG/g DW, 48.5 μg/g DW at 55 °C, and undetectable levels at 80 °C. In addition, the effect of oil-extraction on the stability was assessed in T2 seeds, observing a higher stability when exposed to 55 °C (Hayden et al. 2013).

In 2014, Hayden et al. reported an additional effort to explore the appropriate processing of corn-made vaccines. The study focused on determining if defatting seeds lead to a global increase in antigen concentration due to the loss of lipids in the biomass, with minimum antigen loss during extraction. Corn seeds expressing HBsAg under control of a 3 kb 2× globulin 1 promoter (Hayden et al. 2012) were soaked for 5 days in water to approximately 50% moisture to remove germ and afterwards dried at 37 °C to final moisture of 6–15%. Maize was ground to a fine powder and defatted with hexane treatment or supercritical fluid extraction (SFE). Wafers were made with HBsAg expressing maize to a final concentration of 290 μg/g DW for full fat wafers, 481 μg/g DW for hexane-treated wafersand, and 297 μg/g DW for SFE-treated wafers according to ELISA analyses. Immunoblot analysis

showed antigenicity of HBsAg after treatments. BALB/c mice were arranged in six groups: groups 1–5 were injected with 0.5 μg Recombivax® on day 0 and boosted will full fat wafers, hexane-defatted wafers, SFE-defatted wafers, Recombivax®, or control wafers, respectively, with boosting initiated on days 112 and 126 post-injection. Two wafers were offered for each boost per day during three consecutive days or a single intra-muscular Recombivax® injection in the first day. Group 6 consisted of mice injected with 0.9% sterile saline solution on day 0 and boosted with control wafers. In terms of mucosal immune response, the secretory IgA in feces showed that the SFE-treated group elicited the greatest response, while hexane-treated wafers generated the weakest. Recombivax® produced no IgA response, suggesting that the immune response against HBsAg is generated via the mucosal route. Finally, the systemic immune response was assessed showing that the SFE wafers and Recombivax® treatments generated comparable levels of IgG and IgA in serum (Hayden et al. 2014). In an effort to elucidate the mechanisms behind the differential immune responses observed between the SFE-treated and hexane-treated groups, Shah et al. (2015) biochemically and biophysically characterized VLPs obtained from both treatments. The analyses comprised gel filtration, transmission electron microscopy (TEM), dynamic light scattering (DLS), UV-CD, and fluorescence assays. The size of the VLPs from hexane-treated maize germ ranged from 20 to 30 nm, whereas in the SFE-treated germ a uniform and symmetrical, average size of 20 nm was found. The secondary structure analyzed using UV-CD showed a close similarity between VLPs from the SFE treatment and those produced by yeast. Therefore, there is an association between the method used to prepare the material for oral delivery and the structural and immunogenic properties of VLPs (Shah et al. 2015).

A recent study made by Hayden et al. (2015) assessed the long-term mucosal and systemic immune responses induced by the maize-made HBsAg. Transgenic maize plants were backcrossed to produce a hybrid seed. The HBsAg-expressing germ lipids were extracted by SFE and processed to obtain wafers. The wafers showed a narrow variation on percentage of defatted germ, sugar, and water. An animal study was conducted using BALB/c mice groups subjected to an intramuscular injection of 0.25 μg Recombivax® on day 0 and four boosts with SFE-defatted wafers, Recombivax, or control wafers. The boosts initiated on weeks 13, 15, 47, and 50 post-primary injections; they consisted of two wafers offered per day for three consecutive days or a single 0.25 μg intra-muscular Recombivax® injection administered the first day of boosting. The wafers used for boosts 1 and 2 contained an average of 181 μg/g DW of HBsAg, whereas boosts 3 and 4 contained 567 μg/g DW. The control wafers were obtained from WT plants. IgA, IgG, and total Ig in serum were used to assess the long-term systemic immune response, while IgA in fecal pellets was followed to evaluate long-term mucosal immune response. The results showed that immunization and boosting of mice with highly-expressing HBsAg wafers over 1 year resulted in a superior induction of mucosal and systemic HBsAg-specific IgA relative to the currently commercial, parenterally administered vaccine Recombivax®, which could effectively protect against entry of hepatitis B virus at mucosal surfaces (Hayden et al. 2015).

These detailed studies in the potential of corn as biofactory of hepatitis vaccines are remarkable considering that hepatitis B antigens have been explored since the beginning of the plant-based vaccines technology, but in leafy crops requiring freeze-drying process to stabilize plant biomass and increase antigen concentration (Mason et al. 1992).

Porcine reproductive and respiratory syndrome virus

The disease caused by the porcine reproductive and respiratory syndrome virus (PRRSV) is considered to be one of the most costly pathology affecting intensive pig production worldwide (Pileri and Mateu 2016). Hu et al. (2012) produced the M protein of PRRSV in corn callus using the corn ubiquitin promoter. Corn calluses were transformed by particle bombardment and the transgenic lines reached a production of 5.1 μg/g fresh weight (FW). Protein M antigenicity was evaluated using Western blot and ELISA, observing positive reactivity. BALB/c mice were orally immunized four times at 2-week intervals with 30 mg of dried transgenic corn callus containing 2.6 μg of M protein plus 0.1 mL of Sigma adjuvant system®. Specific anti-M protein IgG was detected in serum of the immunized animals that reached its concentration peak after the third boost. Moreover, a virus neutralizing assay was conducted revealing detectable serum neutralization antibodies. IgA ELISA of fecal samples showed M-protein specific IgA increasing after each boost. Interestingly, the IgA antibodies also showed neutralizing activity. Cellular immune responses were evaluated in stimulated splenocytes of the immunized animals showing a significant production of IFN-γ in the group vaccinated with the plant-made vaccine (Hu et al. 2012).

Influenza virus

Nahampun et al. (2015) developed transgenic maize expressing the nucleoprotein (NP) gene from N3H2 swine origin influenza virus. The NP gene was cloned under control of the endosperm-specific promoter of the maize 27-kDa γ-zein gene. Maize γ-zein signal peptide was

placed upstream of the NP gene, while the C-terminal SEKDEL sequence for endoplasmic reticulum (ER) retention and VSP (soybean vegetative storage protein) terminator were included downstream. *Agrobacterium*-mediated method was used for maize transformation obtaining T1 lines expressing recombinant NP (rNP) in the range from 8 to 25 μg of NP/g of corn seed (DW), which increased to 70 μg of NP/g (DW) in T3 seeds as determined by ELISA. The presence of rNP in maize protein extracts was confirmed by western blot. Evaluations on pig and mouse were conducted to assess the immunogenicity of the corn-made NP. The pig study consisted of two groups ($n = 2$) that were intramuscularly immunized with 16 μg of maize-made NP (MNP) or non-transgenic maize extract. The protein extracts were mixed with adjuvant (Benchmark BioLabs item #70101) at a 4:1 ratio with 5 mL of final volume and administered in the neck. Each pig received a prime and boost on days 0 and 21, respectively. The mouse study consisted of animals immunized three times with 20 μg of MNP in maize protein extracts. The group receiving transgenic maize was subcutaneously primed with maize protein extract along with Imject Alum adjuvant in a 1:1 ratio and the following boosts were orally administered through gavage without adjuvant. The pig study revealed the induction of significant immune response by the MNP administration as shown by high anti-NP titers, while the mouse study also revealed high antibody titers that were comparable to those from the positive control group. The IgA measurements revealed that no response occurred in any of the experimental groups. Further analysis of antibody isotypes showed that IgG2a was higher than IgG1, detecting IL-4 production in splenocytes from immunized animals. Further characterization to determine immune polarization induced by this vaccine is required (Nahampun et al. 2015).

Foot-and-mouth disease virus

Zhang et al. (2011) reported two serotypes of the structural protein VP1 from the foot-and-mouth disease virus (FMDV) fused with CTB and LTB, which were co-expressed in maize. Genes coding for serotypes O and Asia 1 of FMDV were used and expression was under control of the Ubiquitin promoter. The signal sequence KDEL for retention at the Endoplasmic Reticulum was linked to the C terminus of the VP1 (Asia 1) sequence. Plants were transformed by a biolistic method and transgenic lines were selected by the level of expression measured with real-time PCR. The stability in inheritance of the gene was confirmed in T_1–T_3 generations by Southern blot and PCR. Evaluations on protein production and antigenicity remain a pending objective for this plant-based vaccine candidate (Zhang et al. 2011).

Newcastle disease virus

Another important work consisted in the development of a vaccine against Newcastle disease virus (NDV). Guerrero-Andrade et al. (2006) obtained transgenic maize lines expressing the F protein from NDV. Yields of the F protein reached up to 3% of TSP. Chickens immunized with either a primary or a primary-plus-booster inoculation using F-containing maize were fully protected against an NDV challenge. This work is an example of a promissory veterinary vaccine using corn as an antigen producer.

Rabies

Rabies is another important problem for animal and human health, especially in developing countries. Transgenic maize lines expressing the G protein from Rabies virus were generated (Loza-Rubio et al. 2012) with expression levels up to 25 μg of G recombinant protein/g of fresh tissue. Doses of corn containing 0.5, 1, 1.5, and 2 mg of G protein were orally administered to sheep. 30 days post-vaccination, only the sheep vaccinated with 2 mg of G protein showed a significant humoral response. The titers increased from day 30 to day 120. After 120 days, the vaccinated animals were intramuscularly challenged with the Rabies virus showing differential responses. An 83% survival rate was observed for sheep immunized with 2 mg of corn-made recombinant G protein or the commercial vaccine. In contrast a 50% survival rate was observed in the animals immunized with 0.5 and 1.0 mg of recombinant G protein, whereas animals immunized with 1.5 mg of recombinant G protein showed a rate of 66%. This report represents a step forward in the field since it provides evidence on the efficacy of an oral vaccine in a polygastric species (Loza-Rubio et al. 2012).

Swine transmissible gastroenteritis virus

In a series of studies, Streatfield et al. (2001, 2002, 2003) expressed the B-subunit from *E. coli* heat-labile enterotoxin (ETEC) and the S protein from swine transmissible gastroenteritis virus (TGEV) in corn. These antigens were fused to the barley α-amylase signal sequence, which directs protein accumulation in the cell wall, with expression driven by the ubiquitin promoter. Corn was transformed using *Agrobacterium*-mediated approach. The level of LTB in corn increased through targeting and plant breeding from 1.8% of TSP in T_1 up to 9.2% of TSP in T_3 seeds. The antigenicity of LTB was assessed by the GM_1 receptor binding assay, sandwich ELISA, and Western blot, observing positive reactivity. Interestingly the stability of the protein was assessed, observing that the LTB pentamer resists heat treatments at 120 °C for 4 min, with

no changes on the pentamer/monomer ratio when located in the intact matrix of the corn seed. BALB/c mice were orally immunized three times with 50 μg of pure recombinant LTB or with 5 or 50 μg of corn-made LTB. The control group received corn material from a WT plant. The antigens were administered using the mouse chow. Similar levels of specific anti-LTB IgG in serum were observed in the groups vaccinated with pure and plant-made LTB. Anti-LTB IgA levels were evident after 7 days reaching peak responses 1 week after each dose. Both IgG and IgA measurements showed that 5 μg of LTB expressed in corn are sufficient to induce an immune response. Interestingly LTB expressed in corn induced a greater anti-LTB specific mucosal IgA response than pure LTB, preventing gut swelling in mice exposed to 20 μg of LT holotoxin.

On the other hand, the S protein from TGEV was used to orally immunize swine on days 0–10 with wild type corn or transgenic corn containing approximately 2 mg of the S protein. Corn was mixed with milk to obtain an oatmeal-like consistency. A control group received the current commercially available modified live vaccine MLV TGEV on days 0 and 7 by the oral route. Swine were challenged on day 12 with a 2 mL dose of virulent TGEV (Purdue strain, titer $10^{7.6}$ $FAID_{50}$'s per dose) observing that only 50% of swine fed with transgenic corn expressing the S protein developed symptoms in contrast to the piglets receiving the commercial modified live vaccine showing 78% of symptoms rate (Streatfield et al. 2001, 2002, 2003).

In a subsequent work, Lamphear et al. (2002) evaluated different immunization schemes. Swine fed for 4 days with TEGV transgenic corn were fully protected against a TGEV challenge (Lamphear et al. 2002). Lamphear et al. (2004) continued with the characterization of the transgenic maize lines reported by Streatfield et al. (2001) and Lamphear et al. (2002) expressing the S protein of the transmissible gastroenteritis virus (TGEV). In this study, gilts from a low incidence herd and seronegative for TGEV were used. The protocol consisted in the oral immunization with the commercially available modified live TGEV vaccine with a titer of $10^{6.9}$ TCID (tissue culture infectious doses)/50 mL at the day of breeding (115 days before farrowing) and 102 days before farrowing, followed by an intramuscular injection 88 days before farrowing. The animals were fed twice with transgenic material 35 and 14 days before farrowing with 2 kg of corn containing 26 mg of antigen. High serum neutralization titers in the animals fed with the transgenic material were observed in serum and colostrum (Lamphear et al. 2004).

Lamphear et al. (2002) evaluated a fractionation process in maize seeds expressing LTB and TGEV, showing that the LTB antigen is greatly enriched in the germ fraction. The antigen remained active after defatting by hexane treatment since mice immunized with 0.7 mg DW of corn containing 0.33 μg of LTB developed a measurable anti-LTB response (Lamphear et al. 2002).

Therefore, corn-based vaccines targeting enterotoxigenic bacteria are promising tools in the fight against diarrheal diseases and other unrelated pathologies.

Critical analysis of current corn-made vaccines

Antigen biosynthesis capacity

The state-of-the-art indicates that genetically engineered corn is a versatile platform for antigen production that has become one of the preferred crops for oral vaccine development. The use of endogenous promoters and signal sequences has allowed expressing and accumulating significant amounts of recombinant antigens in the corn seed. According to the literature, the highest yields for antigen expression in corn is in the order of 0.5 mg/g corn defatted germ using the globulin 1 promoter and the signal sequence of barley aleurain, which targets the protein to the vacuole (Streatfield et al. 2003). In the context of the molecular farming field corn offers medium yields, considering that in rice the yields are above 1 mg/g of plant material; for instance, CTB is accumulated in rice up to 1.5 mg/g (Nochi et al. 2007). However, the agronomic traits of such crops should be also critically considered before electing the expression platform, since growing rice has a higher water requirement whereas corn offers a high seed yield with a lower water requirement. When compared to leafy crops, corn offers higher yields; for instance, hepatitis B antigens are produced in lettuce at 15.9 μg S-HBsAg/g fresh weight (Czyż et al. 2014) and in tobacco at 10 μg/g FW of VLP-assembled S- or M-HBsAg (Fedorowicz-Strońska et al. 2016). In carrot and lettuce, LTB yields were up to 2–3 μg/g FW (Rosales-Mendoza and Tello-Olea 2015; Martínez-González et al. 2011). In contrast, corn offers yields for hepatitis B virus antigens in the range from 0.2 to 0.5 mg/g DW maize-derived materials (wafers; Hayden et al. 2015). Leafy crops demand biomass processing by freeze-drying to concentrate and stabilize the antigen. The use of corn adds substantial advantages with respect to expression in leaves, roots, and tubers since seeds are more stable, show higher yields, and can be easily processed not requiring freeze-drying.

In another comparison, the yields obtained in transgenic corn are lower when compared to the systems based on transient expression mediated by viral vectors (up to 5 g per kg of fresh leaves) (Marillonnet et al. 2005). However, it should be considered that viral vectors require the purification of the antigens to eliminate bacterial residues.

In contrast, the biomass from corn seeds can be used for oral immunization without complex processing due to the lack of toxic compounds and the high stability of seeds. Since viral vectors offer high yields, their use in corn could open new possibilities. Agroinfiltration is the typical strategy to deliver viral pro-vectors in the form of T-DNAs; however, considering that this approach seems impractical given the anatomy of the seed, viral vector-mediated expression could be achieved by following the approach suggested by Salazar-González et al. (2015). The authors hypothesized that inducible viral pro-vectors could be stably inserted into the plant genome and subsequently induced by ethanol at a convenient plant development stage (in this case when seeds are in their late development stage). This approach is highly attractive since the use of Agrobacterium is avoided, eliminating the need for antigen purification and allowing for the straightforward formulation of oral vaccines. Although yields can be improved, the current expression levels reached in corn under several expression approaches are sufficient to formulate efficacious oral vaccines. Thus it is envisioned that capsules or tablets containing defatted corn powder, which possesses a higher antigen concentration, will constitute appropriate oral formulations.

Immunogenic activity

It is clear that most of the corn-made antigens are correctly expressed since they are antigenic and immunogenic as the native antigens. In general, significant specific IgG and IgA responses (local and systemic) in the listed reports were elicited with transgenic material but further characterization of their potential to induce cellular immune responses is an interesting aspect to consider in future studies. The outlook on the immunoprotective potential of corn-based vaccines comprises few studies in which experimental challenge with the corresponding pathogen has been performed, thus extensive research in this direction is a pending objective.

One advantage of using plant cells or biomass for the oral delivery of vaccines consists in the fact that the plant compounds may account for vaccine immunogenicity by diverse mechanisms. Interestingly zein, a major protein reserve in maize endosperm, is one of the main plant proteins explored for various biomedical applications, such as oral delivery of proteins and peptides, vaccine delivery, DNA transfection, and tissue engineering (Hurtado-López and Murdan 2006; Muthuselvi and Dhathathreyan 2006). Remarkably zein holds a GRAS status and can be converted into different structures such as films, microspheres, nanoparticles, micelles, gels, and fibers (Shukla and Cheryan 2001; Liu et al. 2010; Gong et al. 2011; Xiao et al. 2011). Zeins belong to a class of prolamines with an approximate molecular weight of 40 kDa and are classified as α, β, γ, and δ (Aswathy et al. 2012). Zeins behave as amphiphilic molecules but are considered a hydrophobic protein due to the presence of a large number of uncharged amino acid residues (Sousa et al. 2013). Interestingly, zein is capable of resisting the gastric environment, forms gels, and has mucoadhesive properties. All these attributes make zein a promising material in the development of mucosal delivery systems for drugs, vaccines, and other biomolecules (Patel et al. 2010; Nonthanum et al. 2012; Wongsasulak et al. 2013). Zein has been used as a carrier system for peptides and proteins like insulin in an oral administration study (Beck et al. 1997) and conjugated with folic acid to target activated macrophages (Lee et al. 2013). Zein has been also used in the form of nanoparticles to orally deliver plasmid DNA (Regier et al. 2012). This carrier system has also been tested for oral vaccine delivery by Hurtado-López and Murdan (2006) in simulated gastric and intestinal fluids where a fast degradation of zein was observed; therefore, this approach should be used for routes lacking enzymatic activity, e.g. intramuscular, intranasal, or topical. Hence it may be suggested that the immunogenicity of corn-made vaccines, administered by the oral route and without extensive purification procedures, could be supported/enhanced by the effects of zein and other compounds present in the corn biomass. Other compounds that could favor the immunogenicity of corn-made vaccines include polysaccharides. Further research aimed at characterizing the contribution of such compounds will add valuable data to better understand the immunogenic properties of corn-made vaccines. Studying formulation parameters, such as the effect of particle size and the effect of distinct pills, will also provide insights to maximize vaccine efficacy.

Industrial production

Large-scale production of corn-based vaccines could be performed in greenhouses, which would provide optimal growth conditions, as an alternative to alleviate biosafety concerns related to the use of open pollination species (Keese 2008). The cases of transplastomic plants and transiently transformed plants used for industrial production of vaccines constitute an important precedent for generating a large-scale platform to produce corn-made vaccines (D'Aoust et al. 2010; Su et al. 2015). The implementation of good manufacturing practices during production (which includes biomass production, harvesting, and packaging) will allow to consistently produce and control the corn-based vaccines. Thus regulations on the starting materials, premises, equipment, training, and staff hygiene will be required (WHO 2014). There are two types of industrial processing to transform maize into products:

dry and wet milling (Gwirtz and Garcia-Casal 2014). In the latter relatively pure chemical compound classes of starch, protein, oil, and fiber are obtained. Such products are typically used by the subsequent processes to obtain final products (e.g. sweeteners). In contrast dry milling comprises particle size reduction of clean whole maize with or without screening separation, retaining all or some of the original maize germ and fiber (Brubacher 2002). In the plant-made vaccines area, such well-established maize processing approaches will be useful during production scale-up to an industrial level of the corn-made vaccines. Dry milling is likely the most appropriate approach to obtain corn powders amenable for vaccine formulation with subsequent defatting as proposed by Tacket et al. (2004), and the recovered oil could be used for other purposes.

Concluding remarks

Corn is an attractive host for the expression of antigens in terms of yields, proper protein processing, and versatility for industrial production. The preclinical evaluation of several corn-made vaccines targeting human diseases revealed a promising potential for this technology and one candidate was evaluated in a phase I clinical trial with promising results. In the case of veterinary vaccines, evaluation of candidates in the target animal species have also revealed a promising potential. Therefore, corn plays a key role in the plant-made vaccine field and is expected to render new and attractive vaccines in the short term.

Author contribution statement SRM designed the structure of the review, wrote most of the manuscript and made Fig. 1. CA, CSR and BBH contributed to the data analysis. CA made Table 1. CSR participated in the edition of Fig. 1. All authors discussed the results, read and approved the final version of the manuscript.

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Compliance with ethical standards

Conflict of interest None.

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