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Plant cell factories and mucosal vaccines
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Many advances continue to be made in the field of plant-derived vaccines. Plants have been shown capable of expressing a multicomponent vaccine that when orally delivered induces a T-helper cell subset 1 response and enables passive immunization. Furthermore, a plant-derived vaccine has been shown to protect against challenge in the target host. Increased antigen expression levels (up to 4.1% total soluble protein) have been obtained through transformation of the chloroplast genome. In view of these findings, plant-derived vaccines have been proved as valuable commodities to the world’s health system; however, before their application, studies need to focus on optimization of immunization strategies and to investigate antigen stability.

Introduction
Emerging and re-emerging diseases, the increasing age and size of the world’s population and the threat of biological warfare are challenging the research, development and production arenas of the pharmaceutical industry. There are many effective avenues for the production of recombinant pharmaceutical proteins; however, the main challenge lies in achieving cost-effective production on a large scale. Over the past five years the potential for using plants as bioreactors has become well established. Plant systems offer several advantages: they are economical, using low-cost inputs such as light, water and minerals; they are easily adaptable to large-scale operations (by growing more plants); they possess minimal risk of contamination with potential human pathogens; the products may not require purification and pharmaceuticals produced this way can be given mucosally, hence simplifying delivery and decreasing overall cost.

Plant-derived pharmaceuticals can be categorized into three areas: antibodies, biopharmaceuticals, and vaccines. Plant-derived antibodies, also referred to as plantibodies, have extensive uses in many areas including bioremediation, disease resistance in plants and industrial purification processes. The most advanced application of plantibodies, however, is the production of antibodies for medical use. Stoger and colleagues [1] provided a recent review on plant-derived antibodies.

There have been many reports of biopharmaceutical expression in plants and these proteins have a diverse range of applications [2–9] (see Table 1). In general, however, expression levels of these plant-derived biopharmaceuticals need to be increased before commercial production can be accomplished [10].

In the past 12 years, substantial research has shown that plant-derived vaccines are feasible commodities. Walmsley and Arntzen give a general description of this technology and its early development [11]. This review highlights the advances made in the past three years and the future directions for the development of plant-derived vaccines.

Developing antigen expression
Subunit vaccines comprise specific macromolecules that induce a protective immune response against a pathogen. The use of plants to express antigens for use as vaccines has seen continued interest over the past few years, as witnessed by an increasing number of reports of transgenic plant expression of new antigens and the altered expression of previously reported antigens. A range of different plant and vector systems for the expression of antigens have been investigated and some are summarized in Table 2 [12–20]. Seed-specific production of the B subunit of heat-labile toxin (LTB) by Streatfield et al. [19] achieved LTB expression levels reaching 1.8% total soluble protein (TSP) and two separate maize breeding programs have increased antigen production by fivefold [19] and tenfold [20]. The investigations of Chikwamba et al. [20] regarding expression of LTB in maize are also the first to report the use of particle bombardment for the production of plant-derived vaccines. Immunogenicity was demonstrated for hepatitis B surface antigen (HBsAg)
[12,21], the S protein of transmissible gastroenteritis coronavirus (TGEV) [13,14] and a human immunodeficiency virus 1 (HIV-1) epitope [16], while successful challenge trials resulted after immunization with \( P. \) aeruginosa epitopes [15], the FP1 epitope of foot and mouth disease virus (FMDV) [17] and LTB [20].

Additional antigens expressed in plants include the respiratory syncytial virus (RSV) G and F proteins [22,23], the VP6 protein of rotavirus [24–26], the measles virus (MV) hemagglutinin (H) protein [27], and an epitope from the major surface antigen of \( P. \) falciparum (PfMSP1) [28]. Although the immunogenicity of the plant-derived RSV G and F proteins and the measles virus H protein were demonstrated in animal trials, the immunogenicity of the VP6 antigen and the PfMSP1 epitope were not tested.

The development of plant-derived vaccine technology

Vaccines in clinical trials

The second report of clinical trials carried out with a plant-derived vaccine appeared in 2000 [29]. Transgenic potatoes expressing the Norwalk virus capsid protein (NVCP) under control of the tuber-specific patatin promoter were orally delivered to human volunteers in phase I/II clinical trials. Of the total number of volunteers who ingested the transgenic potatoes, 95% developed significant increases in specific IgA antibody-secreting cells while 20% developed specific serum IgG and 30% developed specific stool IgA. Although the increase in specific serum antibodies was modest, the use of adjuvants, improved assembly of NV-like particles and increased dosage of the recombinant protein may improve the NVCP immunogenicity (see Update).

Plastid transformation

Transformation of the plastid genome offers several advantages for antigen expression, including high-level foreign protein expression, removal of the threat of gene silencing, and transgene containment owing to lack of pollen transmission. Daniell et al. [30] published the first report of a plant-derived vaccine developed through chloroplast transformation. Integration of an unmodified cholera toxin B subunit (CTB)-coding region into the chloroplast genome resulted in the accumulation of up to 4.1% TSP in tobacco leaves. GM1 ganglioside-dependent binding assays showed the chloroplast-synthesized CTB to retain its ability to bind to the intestinal membrane receptor; however, immunogenicity studies were not performed.

Targeting antigens

In attempts to facilitate the harvesting of proteins, antigens have been expressed in a tissue-specific manner in maize kernels [19], tomato fruit [23] and seeds of transgenic tobacco [31]. Targeting of the major glycoprotein (gB) of human cytomegalovirus (HCMV) to transgenic tobacco seeds was previously described [32], and further investigations showed that recombinant gB was almost exclusively deposited in protein storage vesicles in mature tobacco seeds [31].

Fusion proteins

Owing to their minimalistic nature, subunit vaccines are often difficult to detect in a recombinant protein expression system and/or ineffective at inducing the mucosal immune system. Additional proteins are therefore often

### Table 1

| Application | Reference |
|-------------|-----------|
| Anticoagulants | [2] |
| Thrombin inhibitors | [2] |
| Growth hormones | [3] |
| Blood substitutes | [2,4] |
| Collagen replacement | [5] |
| Antimicrobial agents | [6] |
| Treatment and/or prevention of neutropenia | [7] |
| Treatment and/or prevention of anaemia | [8] |
| Treatment and/or prevention of hepatitis | [2] |
| Treatment and/or prevention of cystic fibrosis, liver diseases and haemorrhage | [7] |
| Treatment and/or prevention of Gaucher’s disease | [2] |
| Treatment and/or prevention of HIV | [7] |
| Treatment and/or prevention of hypertension | [7] |
| Treatment and/or prevention of organophosphate poisoning | [9] |

### Table 2

| Plant or vector expression system | Antigen | Reference |
|----------------------------------|---------|-----------|
| Potato                           | Hepatitis B surface antigen (HBsAg) | [12] |
| Potato and tobacco               | N-terminal domain of the spike protein (S) from transmissible gastroenteritis coronavirus | [13,14] |
| Tobacco mosaic virus and influenza virus vector | \( Pseudomonas aeruginosa \) F protein | [16] |
| Potato virus X (PVX) vector      | Human immunodeficiency virus type 1 | [17] |
| Potato                           | VP1 epitope of foot and mouth disease virus | [17] |
| Tomato                           | B subunits of the cholera toxin of \( Vibrio cholerae \) (CTB) | [18] |
| Maize                            | B subunits of the heat labile toxin (LTB) of enterotoxigenic \( Escherichia coli \) | [19,20] |
employed to either improve the detection or immunogenicity of a subunit vaccine. Both Gil et al. [33] and Dus Santos et al. [34] fused antigens to the gene encoding β-glucuronidase (GUS) to increase ease of detection within the plant expression system. In both instances, transformants were selected on the basis of GUS activity. The GUS fusion to both the 2L21 protective epitope from canine parvovirus [33] and protective epitope from FMDV [34] proved immunogenic. Additionally, mice immunized with the GUS–FMDV epitope fusion were completely protected against challenge with the native virus.

Cholera toxin (CT) has been used effectively as a targeting protein within plant-derived vaccines. Nemchinov et al. [35] described tobacco mosaic virus (TMV) expression of a Vibrio cholerae CTB fusion to an epitope (HVR1) from the hepatitis C virus (HCV). Tobacco plants inoculated with the recombinant TMV produced the HVR1 epitope fused to a functionally active, pentameric CTB. The plant-derived CTB–HVR1 reacted with HVR1-specific monoclonal antibodies and sera from individuals infected with virus from four of the major genotypes of HCV. Intranasal immunization of mice with a crude plant extract containing the recombinant CTB–HVR1 elicited both anti-CTB serum antibody and anti-HVR1 serum antibody that specifically bound to HCV-like particles.

An epitope of the rotavirus enterotoxin protein (NSP4) has also been fused to CTB and expressed in potatoes [36]. Expressing a multicomponent CT fusion vaccine in potato expanded this work. The vaccine consisted of the CTB–NSP4 epitope fusion and an ETEC fimbral antigen fusion to the component of CTA that associates the A subunit to the CTB subunit (CTA2) [37]. The two fusion proteins assembled into choler toxin-like structures that retained enterocyte-binding affinity. Orally immunized mice generated detectable levels of serum and mucosal antibodies specific for the native antigen. Elevated levels of interleukin-2 (IL-2) and interferon-γ (IFN-γ) were detected in immunogen-challenged spleen cells from the immunized mice. This indicated the presence of a strong T-helper cell subset 1 (TH1) immune response to the three plant-synthesized antigens. This result was supported by the demonstration of a significant increase in CD4+ lymphocyte numbers. Diarrheal symptoms were reduced in severity and duration in passively immunized mouse neonates following rotavirus challenge. This paper represents the first report of a multicomponent, plant-derived vaccine. This work also demonstrates for the first time passive immunization through a plant-derived vaccine and the induction of a TH1 immune response by an orally delivered, plant-derived vaccine.

**Optimizing delivery**

The vaccination strategy and schedule can have a significant effect on the immune response developed. Lauterslager et al. [38] investigated the efficacy of a potato-derived LTB vaccine in mice. It was determined that oral administration of plant-derived LTB elicits a systemic and local IgA response in parentally primed, but not naïve, animals. This was in contrast to the results reported by Haq et al. [39] and Mason et al. [40] who reported significant specific antibody responses in naïve animals and showed protection from challenge [40]. The authors’ explanations included an inadequate immunization schedule, low antigen dose, low antigen immunogenicity, inability to accurately detect antibodies and interference of tuber material in assays. As tuber materials did not interfere in previous studies performed with the same potato variety [39,40], and unsuccessful challenge trials indicated either low or non-existent antibody titers, this investigation highlights the importance of immunization regime and antigen dosage.

After establishing the immunogenicity of plant-derived MV H protein in mice trials [27], Webster et al. [41] optimized the dose of plant material required to obtain high titer, MV-specific, neutralizing antibodies and examined the boosting of MV H DNA immunization with the plant-derived vaccine. A single-dose DNA inoculation followed by multiple, orally delivered, plant-derived boosters, induced significantly greater quantities of MV-neutralizing antibodies than immunization with DNA or plant-derived vaccine alone. This paper reports the first demonstration of an enhanced immune response to a prime-boost vaccination strategy combining a DNA vaccine with orally delivered plant-derived vaccines.

**Stability and processing of plant-derived vaccines**

Reliable methods are needed to quantify plant-derived antigens and ensure their stability. Lee et al. [42] performed preliminary stability studies. Clover plants expressing the Mannheimia haemolytica A1 leukotoxin 50 fusion protein were harvested and allowed to dry at room temperature and ambient humidity for one to four days. After three days, the clover tissue retained approximately 20% of its initial fresh weight; however, no significant degradation of the fusion protein was observed. Hence, the fusion protein did not require refrigeration for stability. The clover-expressed fusion protein induced an immune response in injected rabbits that recognized and neutralized the native antigen in modified neutral red cytotoxicity assays.

Smith et al. [43] performed a more comprehensive stability study. The quantification of antigenically reactive HBsAg was found to be strongly dependent on the ratio of detergent:cell concentration. A 1–20% w/v sodium ascorbate concentration in the extraction buffer improved the measured levels of monoclonal-reactive antigen 4- to 12-fold. Detergent also influenced antigen stability in cell lysates stored at 4°C. Under optimum conditions
stability was maintained for at least one month, whereas excess detergent rendered the antigen susceptible to proteolytic degradation. Proteolysis was counteracted by the addition of skimmed milk or its protein component; this stabilized the antigen for up to two months. Also, by altering the sodium ascorbate concentration or buffer pH, the proportion of HBsAg displaying the monoclonal-reactive epitopes increased between 8- and 20-fold. Although antigen stability will have to be investigated on a case-by-case basis, it is obvious that simple in vitro manipulations may prove valuable in increasing the immunogenicity and stability of plant-derived antigens.

Castañón et al. [44] investigated minimal processing of a potato-derived rabbit hemorrhagic disease virus (RHDV) vaccine consisting of the VP60 gene. Harvested potatoes were peeled, cut into pieces, lyophilized, powdered, stored and used in trials within three months of collection. Rabbits were primed subcutaneously and boosted intramuscularly with extracts made from the potato powder. The rabbits immunized with the transgenic potato elicited specific antibody responses and were protected against challenge with virulent RHDV.

Conclusions
Evidence for plant expression systems leading to the improved manufacture and delivery of vaccines is in hand. Studies have proven plants capable of expression of many different antigens, all of which have demonstrated immunogenicity when tested and some have been shown to provide protection in model and target animals. It has often been stated that there is a need for increased antigen expression levels in plants; however, targeted expression and plant breeding have alleviated this problem. Preliminary investigations into antigen stability, processing and optimal immunization strategies have been performed. However, there is a need for further in-depth studies of individual antigens before plant-derived vaccines can be used as a commodity. Recently, concerns have been raised regarding the regulation of plant-derived vaccines and the safety of the food chain from contamination with these pharmaceuticals. For this reason the initial concept of edible, food-delivered vaccines needs to be developed into inexpensive, plant-derived, mucosal vaccines that are regulated through prescription and not released like other transgenic plants derived, mucosal vaccines that are regulated through the commercial vaccine, there was a clear indication of the conventional rabies virus vaccine. Three of these individuals showed rabies virus neutralizing antibodies, but none of the five controls showed these antibodies. Although no neutralizing antibody was detected before immunization with the commercial vaccine, there was a clear indication of the potential of the plant-derived rabies vaccine to act as a supplementary oral booster for rabies vaccinations.

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