Expert Opinion

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Delivery of plant-derived vaccines

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Many protein subunit vaccine candidates have been expressed in transgenic plants, and in a few cases the recombinant material has entered early phase clinical or target animal trials. The expressed protein can be purified prior to formulation for any preferred delivery approach. However, there are major cost advantages associated with avoiding protein purification and pursuing the oral delivery of a processed plant product containing the recombinant protein. Grains and dry products that are processed from fresh plant tissues can stably store expressed proteins for extended periods of time at room temperature, making refridgeration unnecessary during storage and distribution. Encapsulation of recombinant proteins in plant tissues guards against their rapid degradation in the gut, therefore facilitating the uptake and induction of appropriate immune responses. Early trial data with plant-based vaccine candidates has shown promising safety and efficacy.

Keywords: edible vaccine, gastrointestinal tract, mucosal target, oral delivery, oral vaccine, plant-based vaccine

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1. Introduction

Research to develop and improve protein subunit vaccines is one of the key approaches being followed to combat infectious diseases. The preferred antigen is typically expressed in a recombinant system. An advantage of these vaccines is that they are well characterised, noninfective and immutable. Thus, they are generally considered to be a relatively safe option for vaccination.

1.1 Parenteral versus oral delivery

Injection is the most common method for vaccine delivery. Although this is the most direct and concentrated form of delivery, which typically results in rapid serum immune responses, it does have some drawbacks. The cost of delivery is a major concern for combating diseases that affect large populations in developing countries. Costs must cover needles, syringes and trained individuals to administer the injections. Despite the use of disposables, safety concerns remain over the potential reuse of contaminated needles. In addition, parenteral delivery may not be the most efficacious way to vaccinate against organisms that infect via mucosal surfaces. Furthermore, injectables must generally be kept cold during storage and distribution. This can add significantly to the cost of a vaccine, particularly if distribution is over a large area.

Oral delivery of vaccines offers an alternative to some of these drawbacks. Needles and syringes are avoided, and the need for medically proficient personnel is reduced. In addition, as delivery is directed to the gastrointestinal tract, oral vaccines are well suited for combating intestinal pathogens. Due to the linked nature of mucosal immunity, oral vaccines may also be good candidates to protect against pathogens that invade other mucosal surfaces.

1.2 Options for oral delivery

To be efficacious, an orally delivered subunit vaccine must be delivered to the epithelial lining of the gut, and, therefore, must be protected against degradation in the gastrointestinal tract. Several options have been explored to protect antigens when

delivered orally, including coating them in biodegradable polymers [1], encasing them in liposomes [2] or proteosomes [3], and expressing and delivering them in recombinant attenuated strains of *Salmonella* [4] or *Vibrio cholerae* [5]. The former three approaches do not pose major safety concerns but do require specific manufacturing processes to coat the antigen; therefore, adding to costs. By contrast, with recombinant attenuated live vectors, the antigen is synthesised in the delivery vehicle, obviating the need for purification and processing. However, as they are derived from pathogens, these delivery vehicles raise safety concerns.

1.3 Advantages of plant systems

Expressing subunit vaccine candidates in plants, and delivering this recombinant material orally, serves to encapsulate the antigen in the transgenic expression host while avoiding the use of live delivery vehicles. Even with an encapsulated antigen it is likely that a greatly increased dose will be needed for oral delivery to achieve a comparable degree of efficacy to injected material. Some orally delivered antigen will be degraded in the gastrointestinal tract, with some of the remaining antigen passing through the gut without reaching an epithelial surface. Still more may fail to be taken up, even though they reach the gut epithelium. Thus, oral delivery may require much more antigen to be administered than is typical for injection. Therefore, low production and processing costs are necessary for oral delivery to be viable for subunit vaccines, particularly in cases where the vaccines must be inexpensive for them to be used.

Generating transgenic plant material is relatively inexpensive once high-expressing lines have been developed. Facility and maintenance costs for plant growth are relatively low compared with those for competing recombinant protein production systems using animal cell cultures, transgenic animals or microbial fermentors. Furthermore, provided edible plant species are chosen for expression, inexpensive food processing type procedures can be used to yield palatable products for oral delivery. Thus, transgenic plants provide a relatively low cost means to generate and deliver the high doses required for oral protein subunit vaccines.

Transgenic plant expression systems also offer the potential for the long-term stable storage of vaccines at room temperatures [6]. This greatly reduces the storage and distribution costs typical for injectables and alternative oral delivery vehicles, such as recombinant attenuated microbes.

2. Plant-based production systems

Plant-based vaccines are subunit vaccines in which selected antigens are expressed in recombinant plant systems. Thus, there should be a clear protein target for a subunit vaccine. This somewhat limits the scope for plant-based vaccines, as antigens that can induce a high level of protection have only been identified for a few diseases so far. Preferably, the selected protein should not vary greatly in sequence between infectious strains, nor should it rapidly accumulate mutations with pathogen evolution. However, as discussed below, some plant production systems can rapidly generate recombinant protein in response to an urgent need. As with other recombinant systems, the emphasis is on expressing high levels of full-length antigens, which retain the structure and immunogenicity of native molecules. To achieve protective efficacy, it may also be important to retain functionality (e.g., receptor binding activity) of expressed antigens.

2.1 Plant expression options

Various tissues of several species have been used to express a variety of antigens in plants [7]. The main tissue options for expression are edible fresh leaves, fruits and vegetables, or edible grains. Recombinant proteins have also been expressed in plant systems designed for secretion. These include growing plants using hydroponics, whereby the foreign protein is fused to a signal sequence to direct secretion from the roots [8], and maintaining tissue cultures in which a signal sequence targets the foreign protein for secretion into the surrounding medium [9].

Any preferred type of transgenic plant material expressing a chosen antigen can be generated by introducing DNA encoding this antigen into the nucleus. This can be achieved using engineered strains of *Agrobacteria*, or by a biolistic approach, for which tungsten or gold particles are coated with foreign DNA and introduced into plant tissue by particle bombardment. There are also two alternatives to nuclear transformation for fresh leaf tissue. Foreign DNA can be introduced into the host plant's chloroplast genome [10]. Alternatively, sequences can be engineered into a plant virus, which can be inoculated onto leaves [11]. In this case, antigens are produced in leaves without generating stably transformed plants.

2.2 Tissues suited to protein purification

One factor in choosing a plant species and tissue for expression is whether the requirement is for an edible product or for a pure antigen that can be formulated for any chosen means of delivery. Any plant tissue can be used to produce an antigen that can subsequently be purified, although some tissues are better suited to this than others. Hydroponics [8] and cell culture systems [9] are obviously suitable for the production of antigens for purification, as recombinant protein is secreted; however, yields are not particularly high, and the scale of production is limited to equipped greenhouses for hydroponics, or incubators for cell cultures. These two approaches have principally been applied to tobacco.

In contrast, high levels of expression have been achieved for several recombinant proteins in leaves and grains. Currently, there is considerable effort towards developing economically feasible purification procedures for recombinant proteins expressed in grains; for example, protocols have been developed for proteins expressed in corn seeds. Three of these corn-expressed proteins, avidin [12], trypsin [13,101] and aprotinin [14], are commercially available. Similar efforts are

underway with rice and barley [102]. Grains are fairly rich in protein and have a relatively simple composition; therefore, they are better suited to recombinant protein purification than most plant tissues.

Fruits and vegetables also have a relatively simple composition but are typically lower in protein content than grains; therefore lowering expression levels and making economic protein purification more difficult. Indeed, these tissues are not considered economically viable expression alternatives to produce recombinant proteins for purification.

Leaves are complex tissues and contain phenolic compounds that make the purification of recombinant proteins very challenging. However, by using chloroplast transformation technology, high levels of antigen expression have been reported in leaf tissues [10]. As each chloroplast has multiple copies of the organelle's genome, and there are many chloroplasts per leaf cell, the transgene copy number per cell is much higher than for nuclear transformation, and is likely to boost expression. Although biolistic approaches to nuclear transformation can also result in many copies of the transgene being integrated, such plant lines are generally avoided as they are susceptible to gene silencing phenomena. At present, chloroplast transformation is largely restricted to tobacco, which is not suitable for the oral delivery of an edible vaccine.

Plant viral expression systems have also been widely applied to express antigens in leaves [11,15]. The incorporation of expressed antigens into viral particles greatly aids purification. The technology is generally used to express peptides or small proteins fused to plant viral surface proteins. Thus, it may be limited in producing large subunit vaccine candidates. Following viral particle recovery, the expressed peptide or protein can be cleaved from the carrier using an engineered protease site.

2.3 Tissues for oral delivery

If the plant tissue chosen for antigen expression is edible or can be processed into an edible form, there is no need for protein purification, and the plant-based vaccine can be delivered orally. Avoiding purification greatly reduces production costs, but high levels of antigen expression are still important to ensure that the necessary antigen dose can be delivered in a manageable amount of plant material. Several groups have experimented with plant tissues for oral delivery of an edible vaccine. The crops tested include corn [16] and alfalfa [17] for delivery to farmed animals, and corn [16], lettuce [18], spinach [19], potato [20,21] and tomato [22] for delivery to humans.

2.4 Achieving high antigen expression

High levels of antigen expression are very important. They make protein purification protocols easier to develop and more economical to run. They are also necessary for prescribed doses of antigens to be consumed orally.

Several strategies have been deployed to achieve high-level recombinant protein expression in plants. These include the genetic approach of using plant germplasm well suited to high-level protein production [23]. Other strategies commonly followed include the use of molecular approaches such as linking antigen-encoding sequences to strong promoter and untranslated leader sequences of plant or plant viral origin [24,25], and attaching signal peptides to direct the accumulation of antigens in subcellular locations where they are stable [25,26]. Constructing protein fusions may also stabilise antigens, particularly peptides. If the fused antigen is to be purified, the carrier protein can be cleaved at an engineered protease site but, for the oral delivery of plant material, the carrier will remain attached. This can be a positive feature, as when using a carrier molecule to direct delivery to a mucosal surface [27-29].

With certain antigens, several alternative plant systems have been used and various expression strategies followed to raise recombinant protein levels. For example, the receptor binding subunit of the heat-labile toxin of enterotoxigenic *Escherichia coli* (Lt-B) has been expressed in several plant tissues, including tobacco leaves [30], potato tubers [21] and corn seeds [24,26]. Antigen expression levels of ≥ 10% total soluble protein have been achieved, corresponding to 0.1% of dry weight [26]. Such levels compare favourably with those achieved for other plant-produced recombinant proteins that are currently being purified for commercial sales [12-14]. Such expression levels are also sufficient to allow the proposed oral dose of ~ 1 mg of this antigen to be delivered in only 1 g of edible plant material.

2.5 Integrity of expressed antigens

Various antigens expressed in plant material have been shown to be full length, as judged by analysis on immunoblots, and some have been subjected to sequence analysis and shown to have the predicted N-terminal amino acids, although signal peptides are not always cleaved [31]. Certain antigens have also been shown to form higher order quaternary structures [20,30] or virus-like particles [32,33] typical for the native molecules. Expressed antigens have also been shown to have native activities, such as receptor binding capability [20,30].

Depending on which subcellular location an antigen is targeted to, it may be glycosylated in the plant expression system. For a particular antigen, this may or may not be a desirable feature, and should be taken into account if signal sequences are to be linked to the recombinant protein. Alternatively, potential glycosylation sites can be mutated in the recombinant expression vector prior to plant transformation. Glycosylation is most likely to be appropriate in expressing viral surface proteins, and plant expression systems have been shown to glycosylate such proteins to a similar extent to that observed for the native proteins [34]. Plant systems do show some differences in their patterns of glycosylation to mammalian systems, but these have not generally affected the activity of expressed molecules [12], although vaccine candidates have not been examined in this regard. In addition, strategies are being developed to overcome these glycosylation differences; for example, using transgenic plants in which enzymes involved in plant-specific glycosylation reactions are knocked out [35], or in which mammalian-specific glycosylation enzymes are added [36].

3. Delivery options for plant-based vaccines

There are three broad delivery approaches for vaccine candidate antigens produced in plant expression systems: purification (prior to formulation and delivery by any chosen means), processing to an edible form for oral delivery, and the oral delivery of harvested plant tissue without further processing.

3.1 Delivery of purified antigen

Standard protein purification protocols can be applied to plant tissues expressing desired antigens. With some tissues, standard fractionation procedures used in the food and feed processing industries provide a good starting point for purification; for example, grains can be milled to separate embryo, endosperm and pericarp fractions, among which the ratio of protein to carbohydrate to fat differs. Thus, if an antigen is expressed in the corn embryo, milling the seed and recovering the embryo (or germ) fraction removes most of the starch, which is in the endosperm. Protein can then be extracted from the germ, and filtration and chromatography procedures can be applied to this extract to purify the desired antigen.

Expression of the antigen as a fusion with an affinity tag or protein with a specific binding activity can greatly ease purification, but may require subsequent cleavage of the fused tag or protein. Clearly, high expression levels aid purification enormously, whatever tissue is chosen for antigen production. Preparation of a pure antigen prior to delivery has not yet been pursued for plant-based vaccine candidates. Rather, the emphasis is towards vaccines for oral delivery. However, a few other recombinant proteins have been purified from plants for commercial production [12-14].

3.2 Delivery of processed plant products

The great potential of plant-based vaccines is to be used not only as recombinant expression systems but also as oral delivery systems. This eliminates the significant costs of protein purification. In addition, the plant material encapsulates expressed antigens, which may facilitate delivery to the surface of the gut.

With the possible exception of grain for farmed animals, oral plant-based vaccines will undergo some form of processing and formulation. The timing and form of the processing depends on the plant tissue chosen for expression, the concentration and stability of the antigen, the target species for vaccination, and any particular specifications required for the final product. Fresh tissues with high water contents, such as leaves and fruits, need to be processed following harvest. At its most simple, this may comprise homogenisation and dehydration to yield a powdered form of uniform antigen concentration. Other tissues, such as grains, beans and vegetable tubers, can potentially be stored prior to processing. The ideal processing technologies to pursue are those that are already used in the food and feed preparation industries, as these are established approaches that yield palatable products. Palatability is particularly important for edible human vaccines; for example, extrusion technologies can be applied to corn seed to generate pellets, flakes or puff-like products [37].

If the concentration of antigen in harvested recombinant plant material is insufficient to allow for oral delivery of a required dose, the antigen must be concentrated during processing. Generating a dry powder from fresh leaves or fruits will concentrate the antigen, but some further fractionation may also be necessary. With corn, milling and separating the germ fraction from the remainder of the seed increases the soluble protein concentration and, provided the antigen is expressed in embryo tissues, simultaneously concentrates it [6]. Concentration of the antigen is particularly important for human vaccines, where, for convenience and public acceptance of products, it will be necessary for the antigen to be delivered in small quantities of processed material (no more than a few grams); for example, an antigen in a corn-based vaccine candidate was concentrated during processing such that only 2 g of a palatable product needed to be delivered to allow adequate dosing in a Phase I clinical trial [38]. At the opposite extreme, large farmed animals could conceivably be administered much greater amounts of processed recombinant plant material.

Expressed antigens must be stable throughout the chosen processing technology. This may require modifications to standard food and feed processing procedures; for example, extrusion processing has been applied to corn grain, whereby material is placed under heat and pressure, and forced to pass through an opening that defines its final shape. The procedure had to be modified to ensure that an expressed antigen was not degraded, while still ensuring gelatinisation of starch to provide a palatable product that is suitable for delivery [37]. In a control run of this procedure, pure antigen spiked onto control corn was degraded. Thus, encapsulation of the antigen in grain clearly served to stabilise it through the process.

Expressed antigens should also be stable for extended periods of time in the final processed delivery forms. Ideally, antigens should be stable at ambient temperatures; indeed, this is one of the recognised advantages of plant-based delivery systems: it makes them particularly good candidates for low-cost animal vaccines, and also for vaccines where widespread distribution to large populations makes cold storage an expensive undertaking. In the case of antigen-expressing corn grain, the recombinant proteins have been shown to be stable during ambient temperature storage over periods of ≥ 1 year [6]. This was shown for a candidate farmed animal vaccine in unprocessed seed, and also for a human vaccine candidate in a processed seed fraction. Encapsulation of antigen in the dry seed matrix is likely to be responsible for the observed long-term stability, as the pure antigens rapidly degrade at ambient temperatures.

For some plant expression systems, processing is necessary to achieve an even antigen concentration; for example, antigen levels seem to vary widely in vegetable tubers from the same harvest [39,40]. Processing allows for homogenisation and uniformity of antigen concentration. A homogeneous

consistency of product and a uniform antigen concentration are important requirements for any processing technology applied to vaccine candidates. Uniformity of antigen concentration was demonstrated for a corn-based vaccine candidate in which the antigen was expressed in a defatted germ fraction [26].

Whatever downstream processing approach is taken, vaccine production processes will need to be completed under conditions of good manufacturing practice (GMP). However, tightly regulated production has not yet been applied to plant-based vaccine candidates. This reflects the focus of most published academic work on proof of principle; demonstrating antigen expression and efficacy in laboratory animals. As the procedures proposed for the processing of plant-based vaccines are modifications of established food and feed processing technologies, it should be fairly straightforward to set up a GMP environment for these vaccines.

For edible plant-based vaccines, these GMP processes should not need to be conducted under sterile conditions. As recombinant plants preferred for oral delivery do not harbour human or target animal pathogens, it is anticipated that they are a safe delivery option. However, starting plant material is clearly not sterile, and the oral delivery of plant-based products should not require these products to be sterile. Holding edible vaccine material to standards expected for processed food products should be appropriate. Clearly, the material must be free of pathogens, but eliminating all bioburden is impractical. Stabilisation may result in the expressed antigen being degraded, or the costs of production would rise to greatly reduce the cost advantages of the vaccines. Maintaining sterility during distribution to widely dispersed populations would be particularly costly, nullifying one of the main drivers for uptake of the technology.

Whether transgenic plant material is processed to yield edible products, or provides starting material for purification of recombinant proteins, it will need to express chosen antigens within defined concentration ranges. This will allow the relevant protocols to be certified for set amounts of recombinant proteins. Therefore, expression levels will need to be fairly stable across different growing locations and plant generations. Although expression may vary greatly during line development, once the genetic background becomes more fixed (e.g., by repeatedly crossing to a production line with favourable growth characteristics), then more stably expressing lines can be maintained. This has been observed for recombinant proteins expressed in corn [12]. Master and working seed banks will need to be defined, whereby working seed is repeatedly drawn from to grow plants for vaccine production, and master seed, representing defined lines, is periodically used to generate new reserves of working seed.

3.3 Delivery of harvested unprocessed plant material

For some farmed animal vaccines it may be possible to use harvested or minimally processed material. To ease delivery, tissues chosen for expression should be standard dietary components for target animal species; for example, the expression of candidate vaccine antigens in corn grain may allow direct addition of vaccines to feed at times appropriate for vaccination. This approach has been followed in small-scale feeding trials with a corn-based vaccine candidate directed against swine transmissible gastroenteritis virus (TGEV). The antigen concentration was sufficient to allow the delivery of desired doses in small enough volumes of grain for them to be added to standard rations [6,16,41]. Within assay parameters, expression of the antigen was uniform in harvested grain, allowing even dosing.

Large-scale production requiring multiple harvests over more than one season, and possibly in multiple growing locations, would result in some variation in antigen levels. Even once a master seed bank has been established some minor expression differences are likely due to variable growing conditions. Thus, it will likely be necessary to set the desired amount of antigen in a given amount of corn material to be delivered to animals at such a level as to require the dilution of vaccine corn from any given harvest with an appropriate amount of control grain. In this way, the amount of material to be delivered for a set antigen dose can remain constant, batch to batch.

4. Uptake and efficacy

There are many examples of antigens being expressed in plant tissues, several of which have been tested by injecting plant extracts into laboratory animals [7]; however, only a few of these have been tested by oral delivery as this requires a higher level of antigen expression to be practical, and many early proof of principle studies have not vigorously pursued high levels of expression. However, several antigens have been expressed at sufficiently high levels to attempt oral delivery, and the results from these studies have been encouraging [7].

4.1 Oral delivery studies

Orally delivered plant-based vaccine candidates have been shown to induce serum and mucosal antibodies in animal and clinical studies [6,16,18,19,21,30,38-40,42]. Secretory IgAs have generally been reported at the intestinal delivery site but have also been recorded at other mucosal surfaces. Oral delivery has also been shown to stimulate the production of antibody secreting cells in the peripheral blood [38-40], indicating the induction of the gut's mucosal immune system. Raised levels of specific cytokines have also resulted from the oral delivery of plant-based vaccines [43]. Most preliminary oral efficacy studies have been conducted in mice, but there are also a handful of reported examples of completed Phase I clinical trials [18,19,38-40]. These studies have provided encouraging efficacy data to support the technology. The vaccine candidates also appear to be safe, at least in the context of these small-scale studies; however, some subjects experienced nausea, most likely associated with early experimental delivery forms, such as raw potatoes [39,40].

A few orally delivered plant-based vaccines have been tested in laboratory animal models for protective efficacy, and results have been encouraging [16,21,42]. In addition, a target farmed animal species (swine) was protected against a viral pathogen (TGEV) in a feeding trial with an edible corn-based vaccine candidate [6,16]. Oral delivery of this vaccine to pregnant sows resulted in lactogenic immunity, with high antibody titres in the colostrums [41]. Furthermore, plant-based vaccine candidates, delivered orally to female mice, have been shown to result in their pups carrying vaccine antigen-specific antibodies, which protect against pathogen challenge [43,44]. Thus, these vaccines induced passive immunity.

4.2 Encapsulation

Encapsulation of antigen in transgenic plant material is likely to be important in protecting it from degradation in the gastrointestinal tract before it can reach the epithelium and induce an immune response. In the case of corn, for example, a protein expressed in the seed and delivered to mice was shown to be present in the faeces, along with peptide breakdown products. By contrast, a corresponding amount of the pure protein delivered orally was absent from the faeces, with even reduced-length peptides being undetectable. It is likely that the matrix of the corn seed allowed the gradual release of this protein. Not surprisingly, the corn-delivered material induced much greater immune responses.

4.3 Strategies to boost efficacy

Some of the early antigens expressed in plant systems have characteristics that favour their recognition at the intestinal epithelium and, hence, the induction of immune responses; for example, Lt-B forms pentamers that bind surface ganglioside receptors on the epithelial surface, and hepatitis B surface antigen molecules assemble into virus-like particles.

Antigens lacking such characteristics may not induce such strong responses following oral delivery. In these cases, it may be feasible to express and deliver them as fusions to targeted molecules such as Lt-B. This fusion technology has been tested using microbially expressed proteins, but the fusions are often easily degraded. Encapsulation in plant material may stabilise them sufficiently to allow delivery to the gut epithelium, and this approach is now being explored [28,29]. A complicating feature of such a strategy is that the carrier molecule will also be involved in inducing an immune response, and some carriers induce a very strong and distinct response (e.g., with regard to T helper cell type 1/2). Although such carriers may be beneficial in inducing responses to fused antigens, they may also drive the response in a way unsuitable for protection against targeted pathogens. Thus, carrier molecules should be chosen with caution.

Another alternative is to co-deliver a protein adjuvant also expressed in recombinant plant material. Although not aiding delivery, such an adjuvant may enhance the immune response to an orally delivered antigen. However, as with a fused carrier, it may also bias the type of response. In addition, the use of fusions, and in particular adjuvants, will likely complicate safety and efficacy studies required to gain regulatory approval.

4.4 Dosing

Most plant-based vaccine candidates have only undergone one or a few feeding studies; therefore, important delivery criteria such as identifying the preferred dosing level and regimen have not been resolved. However, preliminary studies with a few of the better studied examples indicate that continuous or repeated high-level dosing does not necessarily lead to stronger immune responses [6].

5. Applicability of the technology

Some disease targets and susceptible populations lend themselves particularly well to plant-based oral delivery of subunit vaccines. Some promising targets are described in this section.

5.1 Mucosal target

As an oral vaccine favours delivery to the mucosal surface of the intestinal epithelium, orally delivered plant-based vaccines are particularly well suited to combat intestinal pathogens such as enterotoxigenic *Escherichia coli*, shigella, rotavirus and *Vibrio cholerae*.

Due to the possible linked nature of the mucosal immune system, pathogens that must or can invade via other mucosal sites may also be good targets for orally delivered plant-based vaccines. Prominent examples include hepatitis B and HIV.

5.2 Cost drivers

Generally, the principal driver for testing plant-based oral delivery is potential cost savings, in particular so when delivery using injectables is cost prohibitive. Thus, infectious diseases affecting large populations in developing countries make very attractive targets for the technology. Stability data with plant-based vaccine candidates indicates that they can be stored without antigen degradation at ambient temperatures [6]. Avoiding cold chains should facilitate inexpensive distribution, emphasising the suitability of plant-based vaccines for widely dispersed rural communities.

5.3 Stability and bulk-up

The stability of plant-based vaccines during ambient temperature storage also makes them well suited for inexpensive stockpiling. In addition, once transgenic master seed has been developed, plant-based vaccines can be rapidly and inexpensively bulked. The response time to an urgent vaccine need is dependent on the growing time of the chosen expression host. The costs associated with generating more transgenic plant material are those of growth, harvest and processing. The two features of inexpensive long-term storage of stockpiles, and rapid inexpensive bulk-up of further material, make plant-based oral vaccines good candidates for responding to epidemics.

5.4 Limitations

Although plant-based vaccines can be rapidly scaled up, most plant expression systems take many months to generate the first transgenic material from which stocks can be bulked. This may limit their applicability for pathogens in which the major antigenic determinants mutate and/or recombine rapidly to generate new infectious strains, as with influenza. One approach to counter recombination for a disease such as influenza is to produce multiple plant lines, each expressing an alternative common form of target antigens. These could then be stored as master seed ready for bulk-up as necessary, or even stockpiled in a bulk processed form. When required, material from the relevant lines could be appropriately combined to yield the desired vaccine.

Alternatively, the plant expression technology relying on the infection of plant tissues with engineered plant viruses is more receptive to rapidly generating plant material expressing a new version of an antigen. The reduced timeframe reflects the engineering of plant viral genomes rather than those of plants.

5.5 Animal targets

The cost advantages of edible plant-based vaccines make them particularly good candidates for diseases of farmed animals, especially if the plant tissue for expression is a part of the target animal's diet. Corn is a particularly good expression system in this regard, as it is readily consumed by most farmed species. However, the digestive systems of some species, such as ruminants, may prove challenging for oral vaccines, even those encapsulated in plant material. It should be noted that to be cost effective, plant-based vaccines for farmed animals do not need to be fully efficacious. Partial protection leading to decreased levels of mortality and morbidity and an increased rate of weight gain across herds should be a sufficient economic driver to stimulate uptake of the technology. Plant-based vaccine approaches are being explored with several diseases of farmed animals, including transmissible gastroenteritis, rinderpest and foot and mouth disease.

Other potentially good targets for plant-based vaccines are diseases that affect humans but are carried by, and may also affect, farmed and/or wild animals. Examples include rabies and West Nile virus. The cost advantages of delivering plant-based vaccines to animals may provide a means to effectively combat the widespread transmission of such diseases.

5.6 Market entry

Early penetration of markets in both human and animal vaccine areas may be achieved by using plant-based vaccines as boosters to prior injections. The cost savings of avoiding later injections can be considerable, and efficacy expectations are more easily achieved than with stand-alone applications. Examples of human and farmed animal plant-based vaccines, which are being developed as potential boosters, are directed against hepatitis B and TGEV. In the case of human vaccines, substituting later injections with oral treatments may increase compliance.

6. Conclusion

Plant-based expression offers a cost-effective production system for antigens selected for protein subunit vaccines. Facility and maintenance costs are less expensive than for alternative recombinant systems. In addition, provided edible plant hosts are used for expression, vaccines may be delivered orally in suitably processed plant material; this avoids the considerable costs of protein purification. In addition, the oral delivery route is an inexpensive alternative to providing a course of injections.

Plant-based systems have been used to express a wide range of candidate vaccine antigens. Characterisation of several recombinant proteins has revealed high-level expression of full-length antigens that retain biological activities, such as receptor-binding capability. Processed forms are being assessed as alternative delivery vehicles, with expressed antigens shown to be stable in processed plant materials for long periods of time, even when stored at ambient temperatures.

Feeding studies in laboratory animals, using raw or processed forms of plant material, have demonstrated a range of immune responses and, in some cases, protective efficacy. Furthermore, a few plant-based vaccines have progressed into Phase I clinical trials demonstrating the induction of immune responses in human subjects and positive safety data. Similarly, early studies with target farmed animals indicate that oral plant-based vaccines are a safe delivery option that can induce appropriate immune responses and confer protective efficacy.

7. Expert opinion

Oral delivery of subunit vaccines is an attractive reduced cost alternative to injections. However, becasue much of the consumed antigen may not reach the muscosal lining of the gastrointestinal tract, oral delivery will likely require greatly increased dosage and/or protection of antigen from digestive enzymes.

7.1 Advantages of the technology

Recombinant plant expression systems offer the potential for the inexpensive production of sufficient quantities of antigens to meet the requirements for large-scale oral delivery. The plant expression system can also serve to encapsulate expressed antigens; therefore stabilising them during storage and, following oral delivery, guard against their rapid degradation. This is likely to allow intact epitopes to reach the intestinal epithelium and induce immune responses.

7.2 Avenues for future research

For a sufficient oral dose of antigen to be delivered in plant material, high levels of expression must be achieved. Although this has already been accomplished for selected antigens in a few plant expression systems, expression levels are usually

insufficient, or the plant expression host is unsuitable for oral delivery. Thus, over the next few years, there will continue to be considerable effort focused on raising expression levels of candidate vaccine antigens, with an increased focus on practical host plant delivery systems. There is also likely to be increased activity in exploring alternative processed forms for delivery and advancing these into pilot scale production under GMP conditions, thus providing material for further clinical trials.

Moving into later stage clinical and target animal trials will require a substantial financial commitment to the technology. This is likely to be dependent on partnerships between the biotechnology companies developing this technology and pharmaceutical and/or animal health companies. Alternatively, funding agencies or charitable organisations, viewing this as a viable strategy to vaccinate widely against infectious disease agents, may offer large-scale support.

In the short to medium term, animal vaccine candidates may move most quickly through later stage trials, particularly those for farmed animals. This reflects the reduced regulatory burden with animal vaccines, and the strong cost driver for an inexpensive means of delivery.

7.3 Concerns over the technology

Important issues are under discussion concerning plant-based recombinant protein production. Concerns include the contamination of food or feed with transgenic plant material expressing vaccine candidates, and pollination, by such transgenic plants, of plants dedicated for food or feed or of wild relatives. There are also concerns over wild animals consuming transgenic plant parts containing pharmaceuticals, and over seed or residues from these plants being left in the soil and causing environmental problems postharvest. Such concerns have led to calls to abandon plant expression systems for vaccines, restrict the technology to non-crop species and/or use contained facilities or geographically isolated locations. Some technologies, such as using plant viruses to infect leaf tissues [103] or using aquatic plant species for expression [104], are focusing on the use of contained facilities.

Despite concerns, there are strong economic and health drivers for pursuing plant-based vaccines. They are particularly well suited to combat infectious diseases affecting widely distributed rural populations in developing countries, where the costs of distribution and delivery for injectables needing cold storage can be prohibitive. Crops developed as food sources have been favoured in developing edible vaccines. The very characteristics that make them good food sources also favour high-level expression of recombinant proteins and the preparation of products suitable for oral delivery. Favoured species have high protein contents and can be processed into palatable forms. It is no surprise that some of the most promising efficacy data for the oral delivery of plant-based vaccines comes from edible species.

The containment of recombinant plants expressing vaccine candidates is a very important issue to which a lot of attention is being given. Strict regulatory guidelines exist on the production of plant-made pharmaceuticals, including subunit vaccine candidates [105]. Strategies pursued to prevent outcrossing include the maintainence of isolation distances between production fields and any other plants of the same species, and timing planting to offset sexual maturity of the production crop with food crops in the area. Other potential strategies include the use of male sterile varieties that cannot outcross to surrounding populations and integrating transgene sequences into the chloroplast genome, as few, if any, chloroplasts are transmitted in pollen. Production fields are rigorously monitored during the growing season and for extended periods postharvest to guard against the opportune germination of left-over seeds. In addition, expression is assessed in non-target tissues, such as roots, which may be left in production fields. The target is tight production systems that only express in the harvested tissue. There has also been a move towards conducting environmental impact studies to assess the potential consequences of expressed protein remaining in production fields postharvest.

The regulatory framework to contain the production of plant-made pharmaceuticals effectively is largely established and is being developed further. This should allow the plant production systems best suited for recombinant protein expression and oral delivery to be pursued.

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