Review article
Biomanufacturing of protective antibodies and other therapeutics in edible plant tissues for oral applications

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Summary
Although plant expression systems used for production of therapeutic proteins have the advantage of being scalable at a low price, the downstream processing necessary to obtain pure therapeutic molecules is as expensive as for the traditional Chinese hamster ovary (CHO) platforms. However, when edible plant tissues (EPTs) are used, there is no need for exhaustive purification, because they can be delivered orally as partially purified formulations that are safe for consumption. This economic benefit is especially interesting when high doses of recombinant proteins are required throughout the treatment/prophylaxis period, as is the case for antibodies used for oral passive immunization (OPI). The secretory IgA (SIgA) antibodies, which are highly abundant in the digestive tract and mucosal secretions, and thus the first choice for OPI, have only been successfully produced in plant expression systems. Here, we cover most of the up-to-date examples of EPT-produced pharmaceuticals, including two examples of SIgA aimed at oral delivery. We describe the benefits and drawbacks of delivering partially purified formulations and discuss a number of practical considerations and criteria to take into account when using plant expression systems, such as subcellular targeting, protein degradation, glycosylation patterns and downstream strategies, all crucial for improved yield, high quality and low cost of the final product.

Introduction
Ever since the production of the first therapeutic protein in plants more than two decades ago, plant production platforms have matured and yielded the first commercially available plant-made pharmaceuticals for both human and veterinary applications (Grabowski et al., 2014; Sack et al., 2015a; Yoshiola et al., 2012). Within the wide repertoire of therapeutic proteins, antibodies are among the most popular, due to their enormous potential for the treatment of a wide range of diseases such as certain cancers, autoimmune diseases and infectious diseases.

The number and types of antibodies expressed in plants have increased incessantly since the first reports in 1989 (Hiatt et al., 1989), and in recent years, some plant-made antibodies (PMAbs) have been presented as promising therapeutic solutions. For instance, the safety and immunogenicity of personalized antibodies to treat patients with non-Hodgkin’s lymphoma, produced by Large Scale Biology Corporation (McCormick et al., 2008) and Icon Genetics (http://www.icongenetics.com), have been demonstrated in phase I clinical trials. CaroRX® (Plant Biotechnology INC, Hayward, CA, USA), an oral topical solution based on antibodies against Streptococcus mutans to prevent dental caries (De Muynck et al., 2010; Larrick et al., 2001; Ma, 1988; Wycoff, 2005), was evaluated in phase I and II clinical trials in the United States and has been registered as a medical device in Europe (Larrick et al., 1998). The first good manufacturing practice (GMP)-compliant, plant-derived monoclonal antibody (mAb) to undergo clinical testing in Europe was the human P2G12 against HIV-1, which has been shown to be safe and well tolerated in healthy women when administered intravaginally (Ma et al., 2015). Finally, ZMapp, a cocktail comprising three individual mAbs directed against Ebola, was able to reverse Ebola disease in 100% of the infected Rhesus macaques (Qiu et al., 2014). The recent outbreak of Ebola in West Africa brought attention to this antibody cocktail, which was delivered to a handful of patients with their signed consent. In February 2015, ZMapp received approval from the FDA as an investigational new drug, allowing the start of clinical trials in Liberia.

Although mammalian cells (Wurm, 2004) or baculovirus-infected insect cells (Berger et al., 2004) are currently the most used antibody production systems for the majority of applications, the use of transgenic plants for the expression of recombinant antibodies is gaining momentum. Accordingly, several groups compared the potential of PMAbs as antimicrobial agents with their commercial ‘biosimilars’ Zeitlin et al. (1998) compared a humanized antiherpes simplex virus 2 (HSV-2) mAb expressed in mammalian cell cultures with its counterpart expressed in soybean, proving not only the similarity in their stability in mucosal secretions of the human reproductive tract, but also in the efficacy for the prevention of vaginal HSV-2...
Edible plant tissues as a platform for production of mucosal therapeutics

OPI is one of the most promising applications of PMAbs, particularly when considering that plant species with tissues that are generally recognized as safe (GRAS) may have lower purification requirements. Edible plant tissues (EPTs) could even be consumed safely, making them ideal platforms for the production of gastrointestinal therapeutics aimed at oral delivery. Although there are still very few examples of antibodies produced in EPTs, some edible mucosal therapeutics have been investigated and set a precedent.

The concept of edible plant-made pharmaceuticals was first conceived in the early 1990s, when Charles J. Arntzen championed the idea of making vaccines in edible fruits. While visiting Bangkok, he saw a mother soothe a crying baby by offering pieces of banana, inspiring him with the idea to produce vaccines in genetically engineered food. The advantages would be enormous: yearly and local growth of plants would not only significantly reduce costs and avoid the need for refrigerated transportation over long distances, but vaccines in EPTs would also not require medical personnel or the use of syringes, which, apart from their additional cost, can be contaminated and lead to infections (Mason et al., 1996). Unfortunately, several limitations to the concept appeared; among others, the possible development of immunotolerance to target peptides or proteins, which has to be addressed on a case-by-case basis, the difficulty to control dosage requirements and the fluctuating dosage consistency, varying from fruit to fruit, plant to plant and generation to generation. To deal with the two last problems, the idea of directly consuming the fruit was abandoned and substituted by the use of dried or partially purified formulations, for which the dosage could be standardized, while sustaining low production costs (Tokuhara et al., 2013). Particularly, controlled doses can be achieved using lyophilized plant cells as formulation. As an example, the plant biotech company Protalix Biotherapeutics produces lyophilized carrot cells expressing either anti-TRNP antibodies or Glucocerebrosidase aimed for oral delivery (http://www.protalix.com/development-pipeline/overview-development-pipeline.asp). Other noteworthy examples of lyophilized plant cells expressing biotherapeutics for oral applications are reviewed in Chan and Daniell (2015).

Since then, a number of vaccines, antibodies and other mucosal pharmaceuticals have been produced in EPTs for oral treatments (Table 1). One of the first was a vaccine candidate based on the heat-labile enterotoxin B (LTB) subunit of enterotoxigenic Escherichia coli (ETEC) produced in transgenic potato, which protected against an ETEC challenge in animal studies and was also immunogenic in humans during a phase I clinical trial (Haq et al., 1995; Mason et al., 1998; Tacket et al., 1998), the same was shown when it was produced in transgenic corn (Tacket, 2007; Tacket et al., 2004). Many examples for Alzheimer’s disease, allergic diseases, or autoimmune and infectious diseases show that rice is one of the preferred EPTs for therapeutic protein production (Azegami et al., 2015; Takaïwa et al., 2015). A first example is the MucoRice-CTB, an oral vaccine against cholera, which consists of the antigen of the cholera toxin B (CTB) accumulating in rice seed storage organelles. This vaccine was shown to induce both mucosal and systemic immunity in primates (Nochi et al., 2009), and also exhibited cross-reactivity with the LTB of ETEC in piglets challenged with ETEC, when the vaccine was orally applied to the nursing sow (Takeyama et al., 2015). Another rice example for mucosal applications is the MucoRice-ARP1, containing the variable domain of a rotavirus-specific llama heavy-chain antibody fragment, which provided efficient protection against rotavirus disease (Tokuhara et al., 2013). Also, the group of Takeshi Matsumura in Japan focused their interest on expressing canine interferon α in strawberries. In animal trials, they showed that canine interferon-α that was delivered to beagles by adding dried transgenic strawberries to the dog feed had a protective and therapeutic effect against the canine periodontal disease, even at a very low dose (Yoshiola et al., 2012). Currently, research has advanced to the clinical trial phase (https://unit.aist.go.jp/bprl/bpri-pmt/member_e.html).

Several therapeutic proteins expressed in the chloroplasts of lettuce leaves also illustrate the efficiency and low cost of the production of pharmaceuticals in EPTs, even at industrial set-ups (Kwon and Daniell, 2015). Su et al. (2015a) produced acid-α-glucosidase (AAG) in lettuce leaves and showed that their natural encapsulation in the chloroplast was able to avoid the adverse immune reaction occurring during the protein replacement therapy for Pompe disease when assayed in mice. Following the same rationale, Su et al. (2015b) also expressed the coagulation factor IX (FIX), showing the absence of pathogenic antibodies after haemophilia treatment in mice. Moreover, Shil et al. (2014) showed that feeding of mice with bioencapsulated ACE2/Ang-(1–7) significantly reduced endotoxin-induced uveitis (EU).

As mentioned above, Protalix Biotherapeutics is engaged in the development of two new drugs naturally encapsulated within carrot cells, intended for oral delivery. One of them is the glucocerebrosidase (GCD) enzyme PRX-112 for the treatment of Gaucher disease. Feeding experiments with rat and pig as model
animals are shown that active recombinant GCD reached both liver and spleen, the target organs in the Gaucher disease (Shaaltiel et al., 2015). Moreover, the recombinant GCD was found to be safe and well tolerated in all 12 patients in a phase I clinical trial in Israel. The phase II clinical trial is ongoing for this product. The other carrot cell-based protein is the anti-tumour necrosis factor PRX-106 for orally administered, anti-inflammatory treatment. In preclinical studies, PRX-106 alleviated immune-mediated hepatitis and reduced interferon-γ levels in a mouse model; in a phase I clinical trial, PRX-106 has been shown to be safe and well tolerated, to have biological activity in the gut and to induce regulatory T cells.

There are some examples of antibodies produced in EPTs intended for PI at the gastrointestinal tract, two of them developed in our groups. The first one, intended for veterinary use, is the designer IgAs expressed in Arabidopsis thaliana seeds, consisting of a fusion between the anti-ETEC llama heavy-chain-only antibodies and the Fc part of the porcine IgA. These antibodies have shown to efficiently protect ETEC-challenged piglets against postweaning diarrhoea when administered in the form of crushed seeds mixed with the feed (Virdi et al., 2013). The second example consists of IgA antibodies against rotavirus produced in tomato fruits. In this work, minimally processed fruit-derived products (tomato-clarified extract and powder) suitable for oral intake showed anti-VP8* binding activity and strongly inhibited virus infection in in vitro virus neutralization assays (Juarez et al., 2012).

**Table 1** Most relevant antibodies and therapeutic proteins produced in EPTs

| Product | Target pathogen/disease | Plant species/EPT | Clinical status | Expression level | Reference |
|---------|-------------------------|------------------|----------------|-----------------|-----------|
| Capsid protein | Norwalk virus | Potato | Unknown | 10–20 μg g⁻¹ TW 0.23% TSP | Mason et al. (1996) |
| Heat-labile enterotoxin B (LTB) | *E. coli* | Potato, corn | Phase I | NF | Haq et al. (1995) |
| Virus-like particle (VLP) | Hepatitis B, Norwalk virus | Potato | Unknown | 542 ng mg⁻¹ TSP | Huang et al. (2005) |
| MucoRice-CTB (Cholera toxin B subunit) | Cholera | Rice | Animal trials | 30 μg seed⁻¹ | Nochi et al. (2007) |
| MucoRice-ARF1 (Antibody) | Rotavirus | Rice | Unknown | 170 μg seed⁻¹ | Tokuhara et al. (2013) |
| Canine interferon | Gingivitis | Strawberries | Unknown | NF | https://unit.aist.go.jp/bpri/bpri-pmt/member_e.html |
| VEN100 human lactoferrin | Antibiotic-associated diarrhoea | Rice | Phase II | >5 mg g⁻¹ | Bethell and Huang (2004) |
| PRX-112 glucocerebrosidase (GCD) | Gaucher disease | Carrots | Phase I | 2 mg kg⁻¹ | www.protalix.com |
| PRX-106 antenna | Tumour necrosis factor | Carrot cells | Phase I | NF | Shaaltiel et al. (2015) |
| Designer antibodies Antibody | Enterotoxigenic *E. coli* | *A. thaliana* seeds | Animal trials | 0.2% TSW | www.protalix.com |
| | Rotavirus | Tomato | N/A | 41 μg g⁻¹ FW 3.6% TSP | Virdi et al. (2013) |

**Note:** TW is tuber weight, NF is not found, TSP is total soluble protein, TP is total protein, TSW is total seed weight, FW is fresh weight and N/A is not applicable.

**Secretory IgA as a target molecule for oral passive immunotherapy**

Although IgA constitutes only 10–15% of the total immunoglobulin in the blood serum, it is the predominant immunoglobulin class in external secretions such as breast milk, saliva, tears and the mucosal surfaces of the bronchial, genitourinary and digestive tracts. The daily human production of 5–15 g of SlgA into mucous secretions is greater than that of any other immunoglobulin class (Goldsbey et al., 2003). SlgA serves as the first line of defence at mucosal surfaces by neutralizing pathogenic microorganisms. Because of its polymeric form, SlgA can cross-link large antigens with multiple epitopes. Binding of SlgA to bacterial and viral surface antigens prevents attachment of the pathogens to mucosal cells, thus inhibiting viral infection and bacterial colonization. Complexes of SlgAs and antigens are easily entrapped in the mucus and then eliminated by the ciliated epithelial cells of the respiratory and genitourinary tract or by peristalsis of the gut. For this reason, SlgA is the antibody isotope best suited for OPI (Corthesy, 2003, 2010, 2013).

In spite of all obvious advantages that SlgA presents as a therapeutic molecule, its recombinant production has rarely been attempted for commercial purposes, probably due to the technical difficulties associated with the expression, assembly and recovery of a protein complex composed of four different polypeptides [heavy chain (HC), light chain (LC), joining chain and secretory component (SC)] and comprising a total of 10 individual monomers. Under physiological conditions, the biosynthesis of a SlgA complex requires the cooperation between plasma cells, which produce dimeric IgAs (dlgAs), and mucosal epithelial cells that bind the dlgAs via the polymeric immunoglobulin receptor (pIgR). During transcytosis, the pIgR is cleaved, resulting in the secretion of the SlgA with the Iga-bound pIgR piece, called SC, wrapped around the IgA. Although some attempts to produce a correctly assembled SlgA by co-expressing all four different genes in a single mammalian cell type have been successful (Chintalacharuvu and Morrison, 1997), the yields need to be improved and the technology is yet to be developed to achieve a commercially viable product (Renukuntla et al., 2013).
The first attempt to produce a plant-made SigA for PI was the murine hybrid Guy’s 13 (Ma et al., 1995). This technology was later acquired by Planet Biotechnology (Larrick et al., 2001), evaluated in phase I and II clinical trials and registered as the medical device called CaroRx® (Weintraub et al., 2005). Since then, just a few research groups have reported the expression of SigA in heterologous systems. Wieland et al. (2005) successfully expressed chicken SigAs against Eimeria acervulina in Nicotiana benthamiana leaves. More recently, Virdi et al. (2013) produced SigA-like antibodies against ETEC in A. thaliana seeds. Recently, we also demonstrated the relevance of the proper optimization of SigA production in a case-by-case basis, by performing a combinatorial assembly and analysis of 16 versions of SigA against rotavirus transiently expressed in N. benthamiana (Juarez et al., 2013; Sarrion-Perdigones et al., 2011a). Paul et al. (2014) expressed and characterized a human SigA format of the anti-HIV monoclonal antibody 2G12 using both transgenic tobacco plants and transient expression in N. benthamiana as expression hosts.

Because the production of human SigA antibodies at a commercially viable scale in animal cells remains an unsolved technological problem, plant-based platforms, and especially those using EPTs when SigA production is intended for OPI, appear to be the most promising candidates.

**Practical considerations**

As for every therapeutic which is envisioned to be produced in plants, the design of a plant biofactory for antibody production requires multiple considerations involving not only the expression levels of the antibody, but also additional aspects, such as bioavailability, transformation strategy (Obembe et al., 2011), subcellular targeting, protein degradation, glycosylation patterns and downstream strategies, all of them influencing the yield, quality and cost of the final product (Sarrion-Perdigones et al., 2011b).

To date, most of these aspects have been addressed separately, mainly on an empirical basis. Thus, future optimizations will probably require designs that integrate all of them following a global approach.

One of the main challenges of oral delivery of molecules intended to reach the blood stream is their efficient absorption. Upon oral administration, the gut epithelium acts as a physical and biochemical barrier for absorption of proteins, resulting in low bioavailability. Although this remains an unsolved problem, certain approaches, such as chemical modification, use of absorption and penetration enhancers, use of muco-adhesive polymers and covalent conjugation to carrier molecules such as the (nontoxic) CTB enabling recognition of a target receptor, can enhance the oral bioavailability of peptides. However, these approaches, together with the understanding of the tertiary structures of macromolecules, need to be evaluated on a case-by-case basis to efficiently improve the bioavailability of each target protein (Kwon and Daniell, 2015; Renukuntla et al., 2013).

Another important challenge is achieving high expression levels, however, until now a feasible solution resolving this requirement has not been found. Although the transient expression system in Nicotiana benthamiana is the fastest strategy for recombinant protein production, the yields achieved are not sufficient for obtaining the large quantities of antibodies required for PI strategies. Moreover, it is not the most adequate system for production of therapeutic proteins for oral intake of partially purified formulations, because tobacco leaves lack the GRAS status. On the contrary, when using the seeds of stably transformed plants as a production platform, higher yields can be obtained due to their high protein content nature and low water content (Table 1). If even higher amounts are required, these can be achieved by incorporating a process of lyophilization of different plant tissues. By removing the water content, the concentration of the target protein can be increased more than 20-fold (Chan and Daniell, 2015).

Although extracellular secretion is the natural route for antibodies in mammals, targeting antibody chains to specific compartments in the plant cell can result in advantages in terms of stability, yield or downstream processing (Arcalis et al., 2013; De Muynck et al., 2010). Among the different compartments that have been tested as destination for recombinant antibodies (chloroplast, protein storage vacuole, etc.), the secretory pathway seems to be the most convenient route for correct antibody folding and assembly, due to the oxidizing environment required for formation of disulphide bonds, the low abundance of proteases and the presence of molecular chaperones found in the ER (Ma et al., 2003). Antibody chains are targeted to the plant secretory pathway using an appropriate N-terminal signal peptide, which is either a plant signal peptide (De Buck et al., 2012), or the native signal peptide of the Ig (Hiatt et al., 1989; Sainsbury et al., 2008), which works as efficiently in many cases (De Buck et al., 2012; Hiatt et al., 1989; Sainsbury et al., 2008). Once in the cis-Golgi, antibodies can either be efficiently retrieved back to the ER using a C-terminal HDEL retention signal, or secreted into the apoplast, downstream of the secretory pathway (De Muynck et al., 2009; Petruccelli et al., 2006). Although several antibodies have been reported to accumulate well in the apoplast (De Muynck et al., 2009; De Wilde et al., 1998; Düring et al., 1990), retention in the ER is often preferred for yield improvement and for docking plant-specific, Golgi-derived N-glycosylation with β-1,2 xylose and core α-1,3 fucose that could cause immunogenicity in target organisms (Bencúrová et al., 2004; Gomord et al., 2010). However, as reported by De Meyer et al. (2015) and De Meyer and Depicker (2014), the final protein destination of H/KDEL-tagged recombinant proteins is unpredictable and, hence, plant-specific N-glycans cannot be effectively avoided following this strategy. On another front, the progress in plant glycoengineering has made possible to produce antibodies with customized and highly homogenous glycosylation patterns, which can lead to higher product quality and clinical efficacy (Bosch et al., 2013; Castilho and Steinkellner, 2012; Castilho et al., 2012; Steinkellner and Castilho, 2015). Different strategies have been followed to express recombinant proteins with a mammalian-like N-glycosylation pattern, such as the down-regulation of endogenous α-1,3-fucosyltransferase (FT) and β-1,2 xylosyltransferase (XT), the expression of a chimeric form of the human β1,4 galactosyltransferase (GaIT) targeted to a late Golgi compartment (Bakker et al., 2001; Strasser et al., 2009) and the addition of terminal sialic residues, by engineering of the full mammalian N-acetylneuraminic acid (Neu5Ac) biosynthesis pathway (Castilho et al., 2008, 2010; Loos and Steinkellner, 2014). When glycan adaptation strategies were used for production of antibodies, the resulting glycoforms were highly homogeneous and showed a dramatic increase in the antibody-dependent cellular cytotoxicity (ADCC) activity (Whaley et al., 2011) which points out the benefits of plant-to-human modification of the glycan structure.

Recently, a novel and different approach emerged to efficiently get rid of plant-specific glycosylation. GlycoDelete, a strategy previously developed for its use in mammalian cells (Meuris et al.,...
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2014), consists on expressing a plant Golgi-targeted version of the endo-N-acetyl-b-D-glucosaminidase (endoT) from the fungus Hypocrea jecorina in mutant plants lacking N-acetylglucosaminyltransferase-I (GnT1) activity. These mutants only accumulate high-mannose glycans which are substrates for the endoT enzyme. Consequently, the endoT releases the glycan structure from the target protein, leaving the innermost GlcNAc (which is often important to maintain the overall protein fold) attached to the amino acid backbone (Piron et al., 2015).

Even if it is advisable to mimic mammalian glycosylation in injected therapeutic proteins to avoid unwanted antigenicity, when the therapeutic proteins are aimed at oral treatment, antigenicity against the sugars is probably not so determining. Because we daily consume plant-specific glycans in our diet, plant glycans attached to these recombinant proteins are not expected to generate unexpected allergic reactions. Moreover, the concern of regulatory agencies about glycosylation patterns has probably decreased significantly after the commercialization of ELELYSO® (Protalix Biotherapeutics, Carmiel, Israel), the injectable GCD from Protalix Biotherapeutics, produced in carrot cell suspensions, which showed good results in clinical trials in spite of carrying plant-specific xylose and fucose residues (Aviezer et al., 2009; Cox, 2010; Shalatiel et al., 2007).

As stated above, specific glycosylation patterns can also improve the stability of antibodies. Plants are heterologous environments for antibodies, and proteases may affect the integrity of these during both protein accumulation and protein extraction. Antibodies may undergo complete hydrolysis, directly reducing the final yield, or partial degradation, which can alter the integrity and activity of the final product (Benchabane et al., 2008; Faye et al., 2005; Hehle et al., 2015). Together with obtaining a proper glycosylation pattern of the target antibody, other strategies have been reported to increase both the yield and quality (nondegraded) of PMAbs. For instance, it has been proposed that a balanced co-expression of heavy and light chains is another clue factor for achieving high yields, because unassembled antibody chains, which are retained by the ER-resident chaperone BiP, could be degraded via plant ER-associated degradation systems (Liu and Li, 2014). For this, stronger promoters should be used to boost the expression of antibody chains that are limiting the generation of complete antibody complexes. Other approaches are the use of tissue-specific promoters to confine transgene expression to compartments with reduced metabolic activity or targeting proteins to specific cellular organelles. Gene knockout or silencing of plant peptidases is also a tool to take into account if there is a single or only a few target peptidases, which are not essential for plant growth (De Muynck et al., 2010). For the latter, the specific proteases that are active in the targeted time/space must be detected. Activity-based probes are being developed, which react with the active sites of different enzymes – proteases in this case – in an activity-dependent manner. Proteases can then be identified and further knocked out if required (Ilyas et al., 2015; Lu et al., 2015). Finally, the co-expression of recombinant protease inhibitors interfering with endogenous proteases has also been proposed (Benchabane et al., 2008; Robert et al., 2013). Apart from the in planta stability, there is also a high concern regarding antibody stability in the harsh conditions of the gastrointestinal tract when these antibodies are aimed for oral delivery. In this framework, the glycosylation of the antibodies, and more concretely in the hinge region, also plays a key role (Kim et al., 1994; Plomp et al., 2015). Another advantage of PMAbs that falls within this context is that in partially purified formulations for oral delivery, antibodies are protected by the plant tissue matrix. The plant cell wall provides a natural barrier to the harsh conditions of the gastrointestinal gut that can be further improved by targeting the antibodies to different cell compartments (Sack et al., 2015a). This benefit is also applicable for proteins produced in plant cell suspensions, as it is the case for the above-mentioned therapeutic proteins from Protalix Biotherapeutics, which are naturally encapsulated within the carrot cells.

Of course, the use of minimally processed formulations relies on the logical expectation that the edible status of the transgenic plant tissue remains unaltered. However, it has been repeatedly argued that transgenesis leads to unintended effects in the final composition of the plant tissues, such as changes based on the integration of the transgene, biological interactions caused by transgene-encoded proteins or spurious somaclonal mutations (Araki and Ishii, 2015; Heinemann et al., 2011; Schnell et al., 2015). Although unintended effects have become one of the most controversial questions about biological safety of genetically modified organisms (GMOs), risk analysis studies and detailed GMO characterizations performed until now have not revealed any harmful unintended effect (García-Cañas et al., 2011; Ladics et al., 2015). There are several recent publications that assess the edibility of genetically engineered EPTs producing proteins aimed for pharmaceutical use, one of them being of our group (Juarez et al., 2014). In this work, as a way to evaluate the changes in the tomato composition introduced by IgA production, the proteomic and metabolomic profiles of transgenic tomatoes expressing human IgA were analysed and compared with those of non-transgenic fruits. Although this work itself cannot rule out the possibility of harmful unintended effects associated with every single strategy involving IgA production in fruits, the data suggest that the formulations derived from these IgA tomatoes are as safe for consumption as equivalent formulations derived from wild-type tomatoes. A second work, performed by Kurokawa et al. (2013), describes that known rice allergens appear to be up-regulated by genetic modification of MucoRice-CTB, suggesting that MucoRice-CTB has the potential as a safe oral cholera vaccine. These two studies are the first step for confirming the safety of transgenic plants – or their edible tissues – producing recombinant proteins for mucosal applications. When analysing the unintended effects produced in GMOs, it is also useful to take into account a whole-genome and transcriptomic analysis. In this framework, Kawakatsu et al. (2013) conducted an analysis where the genome of a rice line expressing a seed-based edible vaccine containing two pollen allergens was compared with the genome of its host line. In this study, the authors found that the differences between both genomes were minor in comparison with those found when comparing the parental line with other related rice cultivars, pointing out that most of the alterations corresponded to somaclonal variation. Moreover, when performing strand-specific mRNA-Seq, similar transcriptomes were revealed for both transgenic and parental lines, supporting genomic integrity between them.

Another important consideration is how to handle minimally processed plant preparations or partially purified drug substances to control the quality (e.g. batch-to-batch consistency) of the recombinant pharmaceutical products according to GMP compliance. The procedures for the GMP/GACP-compliant upstream production of 2G12 in transgenic tobacco plants have been recently summarized by Sack et al. (2015b). In general, the manufacturing of recombinant biologically active substances in
Plants are emerging systems for the production of recombinant antibodies. Despite all advantages that plants can offer as antibody expression systems, so far, very few antibodies have been subjected to clinical trials (Sack et al., 2015a). There are two main reasons why PMAbs tend to get trapped in the interface between animal trials and commercialization. First, because mammalian systems are already established and have the dominant share of the market, they somehow obstruct the natural development of new expression systems, even if these have striking advantages. Second, the regulatory framework for the field cultivation of genetically modified plants is highly demanding, particularly in Europe, as described in the directive 2001/18/EC and its amendment 2008/27/EC.

In all probability, plant antibody expression systems are not going to replace the mammalian ones. These systems have well-established production protocols for many antibodies, which have already gone through the current GMP production and drug regulations. Production of ‘biosimilars’ in plants will most likely have to go through strong competition to reach the market. Therefore, the market of PMAbs will probably have more opportunities with the production of ‘biobetters’ (therapeutics with enhanced features and/or efficacy) and of complex molecules that cannot be produced in mammalian platforms, such as SlgA or IgM, which can be exploited for mucosal therapies. Plant platforms present benefits as being human pathogen free, ensuring rapid availability when using transient expression systems, providing à la carte glycosylation patterns and having low production and processing costs. Moreover, if EPTs are used, the final cost of manufacturing can be dramatically reduced, because exhaustive purification would no longer be needed for certain applications. Edible plant tissues with GRAS status (such as fruits, seeds and certain leaves) can therefore be utilized as reservoirs of antibodies such as SlgAs for easy long-time storage and enabling quick availability after minimal processing when required for OPI. In the case of seeds, due to their low water content, they can be stored for years without losing antibody efficacy (Azegami et al., 2015; Virdi et al., 2013). If using more watery tissues, such as fleshy fruits and leaves, a step of lyophilization or spray-drying should be included prior to storing (Juarez et al., 2012; Su et al., 2015b). When using partially purified products derived from EPTs, it is important to take into account that an edibility and allergenicity assessment should be performed to ensure that the plant-derived products are convenient for oral use.

Overall, plant expression systems are evolving and finding their niche to be complementary to other established expression systems. In this framework, EPTS – either seeds or dried tissues – that offer the possibility of long-lasting, refrigeration-free storage and low cost of production and downstream processing, will also be a convenient option for developing countries that cannot afford costly therapies and/or prophylaxis for certain infectious diseases.

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References

Araki, M. and Ishii, T. (2015) Towards social acceptance of plant breeding by genome editing. Trends Plant Sci. 20, 145–149.

Arcalis, E., Stadlmann, J., Rademacher, T., Marcel, S., Sack, M., Altmann, F. and Stoger, E. (2013) Plant species and organ influence the structure and subcellular localization of recombinant glycoproteins. Plant Mol. Biol. 83, 105–117.

Aviezer, D., Brill-Almon, E., Shaaltiel, Y., Hashmueli, S., Bartfeld, D., Mizrahi, S., Liberman, Y. et al. (2009) A plant-derived recombinant human glucocerebrosidase enzyme – a preclinical and phase I investigation. PLoS ONE, 4, e4792.

Azegami, T., Itoh, H., Kyono, H. and Yuki, Y. (2015) Novel transgenic rice-based vaccines. Arch. Immunol. Ther. Exp. (Warsz.) 63, 87–99.

Bakker, H., Bardor, M., Molthoff, J.W., Gomord, V., Elbers, I., Stevens, L.H., Bethell, D.R. and Huang, J. (2004) Recombinant human lactoferrin treatment for heterologous multiprotein complexes. Ann. Oncol. 15, 287–301.

Benchabane, M., Goulet, C., Rivard, D., Faye, L., Boulaflous, A., Benchabane, M., Gomord, W. and Michaud, D. (2008) Preventing unintended proteolysis in plant protein biofactories. Plant Biotechnol. J. 6, 633–648.

Bencírová, M., Hemmer, W., Focke-Tejkl, M., Wilson, I.B.H. and Altmann, F. (2004) Specificity of IgG and IgE antibodies against plant and insect glycoprotein glycan structures determined by artificial glycoforms of human transferrin. Glycobiology, 14, 457–466.

Bendandi, M., Marillonnet, S., Kandzia, R., Thieme, F., Nickstadt, A., Her, S., Fröde, R. et al. (2010) Rapid, high-yield production in plants of individualized idiotype vaccines for non-Hodgkin’s lymphoma. Ann. Oncol. 21, 2420–2427.

Berger, I., Fitzgerald, D.J. and Richmond, T.J. (2004) Baculovirus expression system for heterologous multiprotein complexes. Nat. Biotechnol. 22, 1583–1587.

Bethell, D.R. and Huang, J. (2004) Recombinant human lactoferrin treatment for global health issues: iron deficiency and acute diarrhea. Biometals, 17, 337–342.

Bosch, D., Castilho, A., Loos, A., Schots, A. and Steinkellner, H. (2013) N-glycosylation of plant-produced recombinant proteins. Curr. Pharm. Des. 19, 5503–5512.

Broz, A., Huang, N. and Unruh, G. (2013) Plant-based protein biomanufacturing. Genet. Eng. Biotechnol. News, 33, 32–33.

Castilho, A. and Steinkellner, H. (2012) Glyco-engineering in plants to produce human-like N-glycan structures. Biotechnol. J. 7, 1088–1098.

Castilho, A., Pabst, M., Leonard, R., Veit, C., Altmann, F., Mach, L., Glosd, J. et al. (2008) Construction of a functional CHM-sialic acid biosynthesis pathway in Arabidopsis. Plant Physiol. 147, 331–339.

Castilho, A., Strasser, R., Stadlmann, J., Grass, J., Jez, J., Gattinger, P., Kunert, R. et al. (2010) In planta protein sialylation through overexpression of the respective mammalian pathway. J. Biol. Chem. 285, 15923–15930.

Castilho, A., Neumann, L., Daskalova, S., Mason, H.S., Steinkellner, H., Altmann, F. and Strasser, R. (2012) Engineering of sialylated mucin-type O-glycosylation in plants. J. Biol. Chem. 287, 36518–36526.

Chan, H.T. and Daniell, H. (2015) Plant-made oral vaccines against human infectious diseases-Are we there yet? Plant Biotechnol. J. 13, 1056–1070.

Chintalacharuvu, K.R. and Morrison, S.L. (1997) Production of secretory immunoglobulin A by a single mammalian cell. Proc. Natl. Acad. Sci. USA, 94, 6364–6368.

Corthésy, B. (2003) Recombinant secretory immunoglobulin A in passive immunotherapy: linking immunity and biotechnology. Curr Pharm Biotechnol. 4, 51–67.

Corthésy, B. (2010) Role of secretory immunoglobulin A and secretory component in the protection of mucosal surfaces. Future Microbiol. 5, 817–829.

Corthésy, B. (2013) Role of secretory Iga in infection and maintenance of homeostasis. Autoimmun. Rev. 12, 661–665.

Cox, T.M. (2010) Gaucher disease: clinical profile and therapeutic developments. Biologics, 4, 299–313.

Daniell, H., Streathfield, S.J. and Wycoff, K. (2001) Medical molecular farming: production of antibodies, biopharmaceuticals and edible vaccines in plants. Trends Plant Sci. 6, 219–226.

De Buck, S., Virdi, V., De Meyer, T., De Wilde, K., Piron, R., Nolf, J., Van Lerberge, E. et al. (2012) Production of camel-like antibodies in plants. Methods Mol. Biol. 911, 305–324.

De Meyer, T. and Depicker, A. (2014) Trafficking of endoplasmic reticulum-retained recombinant proteins is unpredictable in Arabidopsis thaliana. Front. Plant Sci. 5, 473.

De Meyer, T., Laukens, B., Nolf, J., Van Lerberge, E., De Rycke, R., De Beuckelaer, A., De Buck, S. et al. (2015) Comparison of VH-HC antibody production in Arabidopsis thaliana, Nicotiana benthamiana and Pichia pastoris. Plant Biotechnol. J. 13, 938–947.

De Muynck, B., Navarre, C., Nizet, Y., Stadlmann, J. and Boutry, M. (2009) Different subcellular localization and glycosylation for a functional antibody expressed in Nicotiana tabacum plants and suspension cells. Transgenic Res. 18, 467–482.

De Muynck, B., Navarre, C. and Boutry, M. (2010) Production of antibodies in plants: status after twenty years. Plant Biotechnol. J. 8, 529–563.

De Wilde, C., De Rycke, R., Beeckman, T., De Neve, M., Van Montagu, M., Engler, G. and Depicker, A. (1998) Accumulation pattern of IgG antibodies and Fab fragments in transgenic Arabidopsis thaliana plants. Plant Cell Physiol. 39, 639–646.

Düring, K., Hipp, S., Kreuzaler, F. and Schell, J. (1990) Synthesis and self-assembly of a functional monoclonal antibody in transgenic Nicotiana tabacum. Plant Mol. Biol. 15, 281–293.

Faye, L., Boulaflous, A., Benchabane, M., Gomord, W. and Michaud, D. (2005) Protein modifications in the plant secretory pathway: current status and practical implications in molecular pharming. Vaccine, 23, 1770–1778.

Frenzel, A., Hust, M. and Schirrmann, T. (2013) Expression of recombinant antibodies. Front Immunol. 4, 217.

García-Calía, V., Simó, C., León, C., Ibañez, E. and Cifuentea, A. (2011) MS-based analytical methodologies to characterize genetically modified crops. Mass Spectrom. Rev. 30, 396–416.

Giritch, A., Marillonnet, S., Engler, C., van Eldik, G., Botterman, J., Klimyk, V. and Gleba, Y. (2006) Rapid high-yield expression of full-size IgG antibodies in plants coinfected with noncompeting viral vectors. Proc. Natl. Acad. Sci. USA, 103, 14701–14706.

Goldsbey, R.A., Kindt, T.J., Osborne, B.A. and Kuby, J. (2003) Immunology. New York: W.H. Freeman and Company.

Gomord, V., Fitchette, A.-C., Menu-Bouaouiche, L., Saint-Jore-Dupas, C., Plsson, C., Michaud, D. and Faye, L. (2010) Plant-specific glycosylation patterns in the context of therapeutic protein production. Plant Biotechnol. J. 8, 564–587.

Grabowski, G.A., Golemb, M. and Shaaltiel, Y. (2014) Taliglucerase alfa: an enzyme replacement therapy using plant cell expression technology. Mol. Genet. Metab. 112, 1–8.

Hag, T.A., Mason, H.S., Clements, J.D. and Arntzen, C.J. (1995) Oral immunization with a recombinant bacterial antigen produced in transgenic plants. Science, 268, 714–716.

Helle, V.K., Lombardi, R., van Doleweerd, C.J., Paul, M.J., Di Micco, P., Morea, V., Benvenuto, E. et al. (2015) Site-specific proteolytic degradation of IgG monomonal antibodies expressed in tobacco plants. Plant Biotechnol. J. 13, 235–245.

Heinemann, J.A., Kurenbach, B. and Quist, D. (2011) Molecular profiling — a tool for addressing emerging gaps in the comparative risk assessment of GMOs. Environ. Int. 37, 1285–1293.

Hiatt, A., Cafferkey, R. and Bowdish, K. (1989) Production of antibodies in transgenic plants. Nature, 342, 76–78.
Huang, Z., Elkin, G., Maloney, B.J., Beuhner, N., Armitzen, C.J., Thanavala, Y. and Mason, H.S. (2005) Virus-like particle expression and assembly in plants: hepatitis B and Norwalk viruses. *Vaccine* **23**, 1851–1858.

Ilyas, M., Hörger, A.C., Bozkurt, T.O., van den Burg, H.A., Kaschani, F., Kaiser, M., Belhaj, K. et al. (2015) Functional divergence of two secreted immune proteases of tomato. *Curr. Biol.* **25**, 2300–2306.

Juarez, P., Presa, S., Espi, J., Pineda, B., Antón, M.T., Moreno, V., Buesa, J. et al. (2012) Neutralizing antibodies against rotavirus produced in transgenically labelled purple tomatoes. *Plant Biotechnol. J.* **10**, 341–352.

Juarez, P., Huet-Trujillo, E., Sarion-Perdigones, A., Falconi, E.E., Granell, A. and Orzaez, D. (2013) Combinatorial analysis of secretory immunoglobulin A (sIgA) expression in plants. *Int. J. Mol. Sci.* **14**, 6205–6222.

Juarez, P., Fernandez-del-Carmen, A., Rambla, J.L., Presa, S., Mico, A., Granell, A. and Orzaez, D. (2014) Evaluation of unintended effects in the composition of tomatoes expressing a human immunoglobulin A against rotavirus. *J. Agric. Food Chem.* **62**, 8158–8168.

Kawakatsu, T., Kawahara, Y., Itoh, T. and Takaïwa, F. (2013) A whole-genome analysis of a transgenic rice seed-based edible vaccine against cereal pollen allergy. *DNA Res.* **20**, 623–631.

Kim, H., Yamaguchi, Y., Masuda, K., Matsuura, C., Yamamoto, K., Irinuma, T., Takahashi, N. et al. (1994) O-glycosylation in hiringe region of mouse immunoglobulin G2b. *J. Biol. Chem.* **269**, 12345–12350.

Ko, K., Tekoa, Y., Rudd, P.M., Harvey, D.J., Dwék, R.A., Spitsin, S., Hanlon, C.A. et al. (2003) Function and glycosylation of plant-derived antiviral monoclonal antibody. *Proc. Natl. Acad. Sci. USA* **100**, 8013–8018.

Korting, H.C. and Schollmann, C. (2012) Medical devices in dermatology: topical semi-solid formulations for the treatment of skin diseases. *J. Dtsch. Dermatol. Ges.* **10**, 103–109.

Kurokawa, S., Nakamura, R., Mejima, M., Kozuka-Hata, H., Kuroda, M., Takeyama, N., Oyama, M. et al. (2013) MucorRice-cholera toxin B-subunit, a rice-based oral cholera vaccine, down-regulates the expression of α-amylase/trypsin inhibitor-like protein family as major rice allergens. *J. Proteome Res.* **12**, 3372–3382.

Kwon, K.C. and Daniell, H. (2015) Low-cost oral delivery of protein drugs. *Nat. Rev. Genet.* **16**, 1336–1343.

McCormick, A.A., Reddy, S., Reinf, S.J., Cameron, T.L., Czewinski-Diks, D.K., Vojdani, F., Hanley, K.M. et al. (2008) Plant-produced idiotype vaccines for the treatment of non-Hodgkin’s lymphoma: safety and immunogenicity in a phase I clinical study. *Proc. Natl. Acad. Sci. USA* **105**, 10131–10136.

Meuris, L., Santens, F., Elson, G., Festjens, N., Boone, M., Dos Santos, A., Devos, S. et al. (2014) GlycoDelete engineering of mammalian cells simplifies N-glycosylation of recombinant proteins. *Nat. Biotechnol.* **32**, 485–489.

Nochi, T., Takagi, H., Yuki, Y., Yang, L., Masumura, T., Mejima, M., Nakashini, U. et al. (2007) Rice-based mucosal vaccine as a global strategy for cold-chain- and needle-free vaccination. *Proc. Natl. Acad. Sci. USA* **104**, 10986–10991.

Nochi, T., Yuki, Y., Katakai, Y., Shibata, H., Tokuhara, D., Mejima, M., Kurokawa, S. et al. (2009) A rice-based oral cholera vaccine induces macaque-specific systemic neutralizing antibodies but does not influence pre-existing intestinal immunity. *J. Immunol.* **183**, 6538–6544.

Obecime, O.O., Popoola, J.O., Leelavathi, S. and Reddy, S.V. (2011) Advances in plant molecular farming. *Biotechnol. Adv.* **29**, 210–222.

Paul, M., Reinh, R., Klein, K., Drake, P.M.W., van Doolwee, C., Palbst, M., Windsdor, M. et al. (2014) Characterization of a plant-produced recombinant human secretory IgA with broad neutralizing activity against HIV. *MAbs* **6**, 1585–1597.

Petruccelli, S., Otegui, M.S., Larue, F., Tran Dinhh, D., Fitchette, A.-C., Circoasta, C., Rambo, M. et al. (2006) A KDEL-tagged monoclonal antibody is efficiently retained in the endoplasmic reticulum in leaves, but is both partially secreted and sorted to protein storage vacuoles in seeds. *Plant Biotechnol. J.* **4**, 511–527.

Piron, R., Santens, F., De Paep, E., Depicker, A. and Callewaert, N. (2015) Using GlycoDelete to produce proteins lacking plant-specific N-glycan modification in seeds. *Nat. Biotechnol.* **33**, 1135–1137.

Plomp, R., Dekkers, G., Rombouts, Y., Visser, R., Koelme, C.A.M., Kammeier, G.S.M., Jansen, B.C. et al. (2015) Hinge-region O-glycosylation of human immunoglobulin G3 (IgG3). *Mol. Cell Proteomics* **14**, 1373–1384.

Qiu, X., Wong, G., Audet, J., Bello, A., Fernando, L., Alimonti, J.B., Fausther-Schnell, J., Steele, M., Bean, J., Neuspiel, M., Girard, C., Dormann, N., Pearson, Robert, S., Khalf, M., Goulet, M.-C., D’Aoust, M.-A., Sainsbury, F. and Vezina, L.-P. and Lomonossoff, G.P. (2015) A KDEL-tagged monoclonal antibody is efficiently retained in the endoplasmic reticulum in leaves, but is both partially secreted and sorted to protein storage vacuoles in seeds. *Plant Biotechnol. J.* **13**, 1094–1105.

Rennkun, D., Vadlaupadi, A.D., Patel, A., Bodd, S.H. and Mitra, A.K. (2013) Approaches for enhancing oral bioavailability of peptides and proteins. *Int. J. Pharm.* **477**, 75–93.

Robert, S., Khalf, M., Foulet, M.-C., D’Aoust, M.-A., Sainsbury, F. and Michaud, D. (2013) Protection of recombinant mammalian antibodies from development-dependent proteolysis in leaves of *Nicotiana benthamiana*. *PloS ONE* **8**, e70203.

Sack, M., Hoffbauer, A., Fischer, R. and Stoger, E. (2015a) The increasing value of plant-made proteins. *Curr. Opin. Biotechnol.* **32**, 163–170.

Sack, M., Rademacher, T., Spigel, H., Boes, A., Hellwig, S., Drossard, J., Stoger, E. et al. (2015b) From gene to harvest: insights into upstream process development for the GMP production of a monoclonal antibody in transgenic tobacco plants. *Plant Biotechnol. J.* **13**, 1094–1105.

Sainsbury, F., Lavoie, P.-O., D’Aoust, M.-A., Vézina, L.-P. and Lomonossoff, G.P. (2008) Expression of multiple proteins using full-length and deleted versions of cowpea mosaic virus RNA-2. *Plant Biotechnol. J.* **6**, 82–92.

Sarion-Perdigones, A., Falconi, E.E., Zandalinas, S.I., Juarez, P., Fernandez-del-Carmen, A., Granell, A. and Orzaez, D. (2011a) GoldenBraid: an iterative cloning system for standardized assembly of reusable genetic modules. *PloS ONE* **6**, e21622.

Sarion-Perdigones, A., Juarez, P., Granell, A. and Orzaez, D. (2011b) Production of antibodies in plants. In: *Antibody Expression and Production (Cell Engineering)*, Vol. 7 (Al-Rubeai, M. ed), pp. 143–164. Dordrecht: Springer.
engineered plants: considerations for pre-market assessments. Transgenic Res. 24, 1–17.
Shaaltiel, Y., Bartfeld, D., Hashmuviel, S., Baum, G., Brill-Almon, E., Galili, G., Dym, O. et al. (2007) Production of glucocerebrosidase with terminal mannos glycan for enzyme replacement therapy of Gaucher’s disease using a plant cell system. Plant Biotechnol. J. 5, 579–590.
Shaaltiel, Y., Gingis-Velitski, S., Tzaban, S., Fiks, N., Tekoah, Y. and Aviezer, D. (2015) Plant-based oral delivery of beta-glucocerebrosidase as an enzyme replacement therapy for Gaucher’s disease. Plant Biotechnol. J. 13, 1033–1040.
Shi, P.K., Kwon, K.C., Zhu, P., Verma, A., Daniell, H. and Li, Q. (2014) Oral delivery of ACE2/Ang-(1–7) bioencapsulated in plant cells for oral tolerance induction in hemophilia A. J. Clin. Immunol. 34, 2429–2437.
Weintraub, J.A., Hilton, J.F., White, J.M., Hoover, C.I., Wycoff, K.L., Yu, L., Larrick, J.W. et al. (2005) Clinical trial of a plant-derived antibody on recolonization of mutans streptococci. Caries Res. 39, 241–450.
Whaley, K.J., Hiatt, A. and Zeitlin, L. (2011) Emerging antibody products and vaccines targeting enterotoxigenic Escherichia coli infection. Vet. Immunol. Immunopathol. 140, 123–134.
Zeitlin, L., Olmsted, S.S., Moench, T.R., Co, M.S., Martinell, B.J., Paradkar, V.M., Russell, D.R. et al. (1998) A humanized monoclonal antibody produced in transgenic plants for immunoprotection of the vagina against genital herpes. Nat. Biotechnol. 16, 1361–1364.

Takaawa, F., Wakisaka, Y., Takagi, H. and Hiroi, T. (2015) Rice seed for delivery of vaccines to gut mucosal immune tissues. Plant Biotechnol. J. 13, 1041–1055.
Takeyama, N., Yuki, Y., Tokuhara, D., Orokku, K., Meijima, M., Kurokawa, S., Kuroda, M. et al. (2015) Oral rice-based vaccine induces passive and active immunity against enterotoxigenic E. coli-mediated diarrhea in pigs. Vaccine. 33, 5204–5211.
Tokuhara, D., Alvarez, B., Meijima, M., Hiroiwa, T., Takahashi, Y., Kurokawa, S., Kuroda, M. et al. (2013) Rice-based oral antibody fragment prophylaxis and therapy against rotavirus infection. J. Clin. Invest. 123, 3829–3838.