Role of plant expression systems in antibody production for passive immunization

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ABSTRACT Passive immunization is a method to achieve immediate protection against infectious agents by administering pathogen-specific antibodies. It has proven to be lifesaving for many acute infections, and it is now also used for cancer treatment. Passive immunization therapies, however, are extremely expensive because they require large amounts of specific antibodies that are produced predominantly in mammalian expression systems. The cost for manufacturing plant-made antibodies is estimated to be comparatively low since plant production systems require relatively less capital investments. In addition, they are not prone to mammalian pathogens, which also eases downstream processing along with making it a safe expression system. Moreover, some of the recent developments in transient expression have enabled rapid, cGMP (current Good Manufacturing Practices) compliant manufacturing of antibodies. Whether lower production costs will be reflected in a lower market price for purified antibodies will be known when more plant-produced antibodies come to the market. Promisingly, the current molecular techniques in the field of in planta expression have enabled high-level production of a variety of antibodies in different plant organs, like roots/tubers/fruits, leaves and seeds, of a variety of plants, like potato, tobacco, maize, rice, tomato and pea, providing a very wide range of possible plant-based passive immunization therapies. For instance, the production of antibodies in edible tissues would allow for a unique, convenient, needle-less, oral passive immunization at the gastric mucosal surface. The technological advances, together with the innate capacity of plant tissues to assemble complex antibodies, will enable carving a niche in the antibody market. This non-exhaustive review aims to shed light on the role of plants as a flexible expression system for passive immunotherapy, which we envisage to progress alongside the conventional production platforms to manufacture specialized antibodies.

KEY WORDS: molecular farming, in leaf production, in seed production, plantibodies, disruptive technology

Introduction and scope of passive immunization

One of the most important arsenals in fighting infectious diseases is the antibody or the protein called immunoglobulin (Ig). After natural infection or vaccination, the immune system is primed to produce pathogen-specific antibodies, which in turn provide protection against subsequent infection by the same pathogen (Durandy et al., 2009). As an alternative to vaccination, which is predominantly prophylactic and needs to be administered prior to the contingency of infections, 'immediate protection' can be achieved by direct administration of disease-specific protective antibodies. The administration of disease-specific antibodies either prophylactically or post-exposure is called passive immunization (Gonik, 2011, Naz and Rajesh, 2004, Raab, 2011, Zeitlin et al., 2000, Zeitlin et al., 1999). The immediate protective effect of passive immunization is temporal, lasting up to months (Zeitlin et al., 2000), and is suitable for emergencies like protection of newborns against vertical transmission of viruses from the mother, protection against biological warfare or emerging diseases, protection of elderly and immune-compromized patients, etc.

The use of passive immunization for the prevention of infectious diseases in human subjects can be traced back to the 1800s, when

Abbreviations used in this paper: CaMV, cauliflower mosaic virus; cGMP, current good manufacturing practices; Fc, fragment crystallizable; GM, genetically modified; Ig, immunoglobulin; ER, endoplasmic reticulum; P35S, 35S promoter; PB, protein body; PSV, protein storage vacuole; mAb, monoclonal antibody; ScFv, single chain variable fragment; VHH, variable fragment of heavy chain only antibodies.
antibodies were sought from animal sera like rabbit and horse (Eibl, 2008). However, infusion of animal serum was not tolerated, since it leads to the immune complication called ‘serum sickness’, particularly after repeated use. Later, human antibodies (from human convalescent sera) were available and passive immunization was largely used during World War I, to treat tetanus, diphtheria and pneumococcal disease. By the 1930s and 1940s, the advancement of fractionation techniques resulted in the separation of plasma proteins and the opportunity to administer a stable biological fraction. Such fractionated serum was used to treat poliomyelitis, mumps, measles, pertussis and even hepatitis A (Raab, 2011). With the emergence of antibiotics and vaccines, the use of passive immunization declined (Berghman et al., 2005, Hsu and Safdar, 2011), but when immediate protection is needed or when vaccines are not a choice, passive immunization is still applied (Holliger and Hudson, 2005, Naz and Rajesh, 2004). A nice example is passive immunization with anti-rabies polyclonal antibodies after being bitten by a rabid animal in regions where rabies is endemic (Both et al., 2012). Moreover, antibiotic replacement is actively sought given the risk of introducing resistant bacterial strains. It is anticipated that passive immunization will regain popularity and might reduce the dependency on the traditionally used antibody therapy (Berghman et al., 2005, Oleksewicz et al., 2012, Zeitlin et al., 2000).

The antibody technology has been developed in leaps and bounds. Specific high quality antibodies against one specific epitope, called monoclonal antibodies (mAbs), can be produced using hybridoma technology, involving the fusion of spleen cells from an immunized mouse (or rat) with immortalized myeloma cells (Köhler and Milstein, 1975). Today, this platform contributes to some of the major demands of monoclonal antibodies in research and diagnostics. However, for therapeutic application, the use of murine IgG is discouraged due to possible side effects arising from an immune response to foreign IgGs. Hence, for human application, humanized or at least partially humanized antibodies are needed. The production of human hybridomas has been difficult; instead B cells from transgenic mice with the human IgG gene repertoire are used to generate humanized Ig (Brüggemann et al., 1989, Schirrmann et al., 2008). There are some drawbacks to the hybridoma technology for passive immunization, as it is inefficient to produce antibodies against toxoids and conserved antigens, and it requires immunization and the development of an in vivo immune response (Schirrmann et al., 2008). In vitro technologies, like phage display, have liberated it from these constraints, and the antigen-binding antibody domains can be quickly sequenced (Smith, 1985). Recently, the in vitro technology for the development of human mAbs directly from a single B cell derived from an amnue of peripheral blood mononuclear cells has been well established (Tiller et al., 2008). This is done by sequestering the specific antibody encoding genes using state of the art molecular biology tools. These gene sequences can then be optimized for expression in various systems. This technique has greatly contributed to the discovery and development of antibodies, in particular for HIV neutralizing antibodies from infected donors (Andrabi et al., 2012). The technological developments have thus given a boost to the passive immunization field and aim to provide part of the solution for the increased disease burden and emergence of new pathogens.

Passive immunization is currently also used to treat non-infectious diseases, like cancer (e.g. FDA approved Bevacizumab and Cetuximab for colon cancer, Alemtuzumab for chronic lymphocytic leukemias), autoimmune diseases (e.g. FDA approved Adalimumab for rheumatoid arthritis) and Alzheimer (Bapineuzumab and Solanezumab are being evaluated in clinical trials) (Casadevall et al., 2004, Holliger and Hudson, 2005, Zeitlin et al., 2000). Antibodies specific for drugs, like cocaine and nicotine, are also investigated to regulate and control drug abuse by preventing their access to the brain, and thus regulating the drug-induced effects. Currently ongoing clinical trials will provide more information about this novel application of antibody therapy (Kosten and Owens, 2005).

Mucosal surfaces comprising the respiratory, gastrointestinal, reproductive and genitul tract are the gateways for most pathogenic infections. Most vaccines fail to achieve a systemic, as well as a mucosal immune response. Topical application of antibodies at the mucosal surface can immediately prevent pathogenic invasion at the first port of entry (Corthésy, 2003, Corthésy and Spertini, 1999). A mixture of three mAbs (2G12, 2F5 and 4E10), as a mAbGel is being evaluated in a clinical trial as vaginal microbicide for the prevention of HIV in heterosexual couples. Development of such microbicides against sexually transmitted diseases is very important in preventing unsafe sex-related death and disability (Whaley et al., 2011).

When it comes to the recombinant production of antibodies, mammalian cell cultures have been readily better suited to obtain highly functional proteins with proper glycosylation, as compared to other prokaryotic and eukaryotic platforms. Therefore, the majority of clinical therapeutic antibodies are produced in mammalian cells, despite the high production costs. The yield of the antibodies produced in mammalian cells has increased more than 10-fold (more than 5 g/L) since the 1980s (Wurm, 2004). This high production level was achieved through high cell densities, high antibody expression per cell and improvement in chromosomal integration of antibody encoding genes. However, stable transformation of mammalian cells is a lengthy process. As an alternative, transient expression with viral promoters has empowered production of hundreds of milligrams of antibodies (Schirrmann et al., 2008). Nevertheless, the mammalian system is still expensive and has become the limiting factor for widespread passive immunization. Expression in transgenic plants might be a solution to effectively scale up therapeutic Igs, and lower the production costs. The recent developments in plant transformation tools have enabled effective production of almost all kinds of antibody and antibody formats, some of which, like the complex secretory IgA (SlgA) for mucosal passive immunization, have thus far been successfully manufactured only in plants (Paul and Ma, 2011, Virdi et al., 2013, Xu et al., 2012). Further, Igs with engineered human-like glycosylation can also be produced in plants (Webster and Thomas, 2012). In the next section we describe the plant as antibody production platform in detail, to highlight how plant expression platforms can help in sharing the load of antibody production together with the established mammalian platform. We believe its particular merits will lie in applications (i) when the antibody production cost has to be minimal, (ii) when Igs with novel complex formats are needed, (iii) for rapid production in case of emergencies like bioterrorism and (iv) in instances where plant tissues can act as a delivery system, particularly for veterinary applications.

**Antibody expression in plants**

The first proof of concept for functional antibody production in plants was provided in 1989, when two transgenic tobacco plants, each expressing light or heavy chains, were produced by...
Plant-based antibody production

Plant expression systems are an attractive platform for the production of antibodies, for several reasons. Predominantly due to the possibility of production scale-up at a fraction of the costs compared to conventional systems. It has been estimated that for plant-based antibodies expressing up to 1% of total soluble protein, the production cost would be 0.1% of that of the mammalian cell culture system and up to 2-10% of that of microbial systems (Chen et al., 2005). Another advantage is that many plant species have a ‘generally regarded as safe’ status, since they do not contain mammalian viruses or pathogens, or produce endotoxins. The ease of purification and downstream processing of plant-made antibodies is often postulated to result in a low cost of the final product, which can be applied parenterally, topically or orally (Xu et al., 2012). Then there is the merit of speed: using the established state of the art tobacco leaf-based transient expression system, bulk quantities of antibodies can be manufactured in a record time as compared to any other established expression system (Castilho et al., 2011). Moreover, the developments in glyco-engineering of plants has made it possible to produce antibodies with desired glycoforms. Modification of glycans has also been perfected in comparative expression systems like mammalian cell cultures, but it has been seen that glyco-engineered plants have a much higher degree of glycan homogeneity (Castilho et al., 2011, Olinger et al., 2012). Having a higher degree of desired glycosylation can lead to higher product quality and clinical efficacy; as demonstrated in case of h-13F6, an anti-Ebola virus monoclonal antibody (Olinger et al., 2012, Zettlin et al., 2011). The plant-derived version of h-13F6, bearing the complex N-glycosylation and devoid of the core fucose, showed higher potency than the original version derived from murine cells.

In conjunction, expression in edible plant tissues like tubers, roots and seeds, is anticipated to open a new avenue for passive immunization against enteric diseases of farm animals, which so far has been impossible due to the high costs using antibodies produced through conventional systems (Floss et al., 2007). Additional benefits of in seed expression of antibodies are the storage at ambient temperature and ease of transportation, which can enable decoupling of the scale-up in the field and the downstream processing (Khan et al., 2012) and which can reduce the burden of cold chain maintenance during shipping.

Different plant-based expression systems

Several plant systems have been explored to achieve an abundant overall production of heterologous proteins with a low capital investment (reviewed in Xu et al., 2012). Antibodies have been produced in moss (Decker and Reski, 2008), microalgae (Franklin and Mayfield, 2005, Mayfield and Franklin, 2005), duckweed (Cox et al., 2006), plant cells (Hellwig et al., 2004), organ cultures (Hellwig et al., 2004, Sharp and Doran, 2001) and land plants – both monocots (like maize) and dicots (like Arabidopsis, tobacco, potato, soybean, alfalfa) (De Muynck et al., 2010). All autotrophic plants have a relatively similar cellular machinery and glycosylation pattern, and most can be transformed by Agrobacterium-mediated transformation or by particle bombardment, two methods of which the former is preferred, as it tends to introduce a low T-DNA copy number in the plant genome (Cheng et al., 2004, Ko and Koprowski, 2005). After the rather time-consuming process of production and selection of homozygous plant lines showing stable antibody expression at a high level, further scale-up for future bulk production can be done with existing farming infrastructure. Alternatively to the development of stably expressing transgenic plant lines, quick protein production can be obtained by transient expression using Agrobacterium infiltration (Kapila et al., 1997) or viral vector expression systems (Gleba et al., 2005, Mor et al., 2003), each having its own benefits.

Depending on the antibody or antibody fragment to be expressed, the downstream processes, the delivery system, etc., the most applicable plant expression system needs to be chosen. However, currently leaves, followed by plant culture systems (hairy roots, cell culture, etc.) and seeds, are most widely used, given the obtained quality and quantity of the recombinant antibody. Here, we review the in leaf and in seed expression systems. For more information about plant culture systems, we recommend the review from Xu et al. (2011).

In leaf antibody expression

The most important motivation for antibody production in leaves is the capacity to scale-up and obtain a high amount of biomass through multiple harvests per year. Until 2010, more than 50% of plant-made full length antibodies were expressed in leaves (De Muynck et al., 2010). Of the plant species that have been explored for in leaf production of antibody and antibody fragments, tobacco (mostly Nicotiana tabacum and N. benthamiana species) has emerged as one of the leading platforms (Paul and Ma, 2011), as it can produce yields of up to 300 tons of biomass per acre, can be conveniently grown in field as well as in greenhouses, and is not regarded as a feed/food plant. More importantly, genetic manipulations of tobacco are easy, and antibody genes can be introduced both stably in the nucleus via Agrobacterium-mediated transformation, or transiently by Agrobacterium infiltration or by viral vectors (Whaley et al., 2011).

One of the most successful examples of a tobacco-made antibody for passive prophylaxis is the secretory antibody CaroRx™ from Planet Biotechnology (www.planetbiotechnology.com), which is now available within the European Union for the prevention of dental caries. Recombinant production of these hetero-decameric SlgAs is commercially not feasible by conventional production systems, and to date plants remain the only viable platform for the production of SlgAs (Paul and Ma, 2011). All elements needed for the expression of SlgAs are introduced in one plant line by successive crossing of individual tobacco lines expressing the light and heavy chains, the joining chain, and the secretory component (Ma et al., 1995, Wycoff, 2005). The combination of stable nuclear transformation and classical breeding has proven to be a robust system for the production of such complex antibodies (Paul and Ma, 2011). Based on the same strategy, Planet Biotechnology has developed two other promising products: DoxoRx™ for drug-induced alopecia, a common side effect of cancer therapy, and RhinoRx™
for the treatment of colds.

Another successful example of stable expression of antibodies in tobacco leaves is the 2G12 anti-HIV antibody. This antibody was isolated from human sera and has drawn the attention due to its characteristic property to neutralize various isolates of the HIV virus (Paul and Ma, 2011, Trkola et al., 1996). This antibody was expressed under the control of a constitutive promoter and tissue-specific promoters in seeds of maize (Rademacher et al., 2008, Ramessar et al., 2008) and Arabidopsis (Loos et al., 2011a), and in tobacco leaves. The 2G12 antibody-producing tobacco plants were grown in a contained greenhouse in compliance to cGMP procedures at Fraunhofer IME, Aachen, Germany (Paul et al., 2011), and immediately purified on site by a customized downstream protocol. Growing plants in regulated greenhouse conditions is advantageous as it prevents the chance of gene flow to the environment through escape of pollen and dispersal of seeds. The regulated environmental conditions within the greenhouse, like temperature, humidity, etc., also enable maintenance of quality and quantity from one batch to another, as it has been noted that biotic and abiotic factors do affect the yield of plant-made recombinant proteins (Paul and Ma, 2011). Production in greenhouses increases the costs as compared to field-grown therapeutic antibodies, but on the other side, it paves the way for establishing regulatory procedures (Paul and Ma, 2011, Paul et al., 2011).

There are several excellent transient expression systems developed that enable large-scale production of recombinant proteins, including antibodies, in about eight days after cloning of the target gene DNA. Some of these include virus-based expression systems, like magnICON® (Gleba et al., 2005), Gemini (Huang et al., 2010) and Geneware (Pogue et al., 2010); and engineered vectors for Agrobacterium-infiltration, like the pEAQ system, in which the T-DNA also bears the p19 viral silencing suppressor (Sainsbury et al., 2010, Sainsbury et al., 2009, Voinnet et al., 2003). The possibility of high-scale antibody production in the limited space of greenhouses and over a short period of time enables multiple rounds of production. This also eases the downstream processing and enables a high recovery of purified antibody. Companies and institutions like Medicago (Quebec city, Quebec, Canada), Kentucky BioProcesses (Owensboro, Kentucky, USA), Texas A&M (college station, Texas, USA), Fraunhofer (Newark, USA) and Icon Genetics (Bayer, Halle, Germany) have established infrastructures for large-scale automated systems to grow tobacco plants in the greenhouse, infiltrate/infect, and harvest the protein (Whaley et al., 2011). Once the antibody genes are isolated and cloned, within two weeks the antibody can be administered to the patient (Paul and Ma, 2011, Whaley et al., 2011). A disadvantage of the transient system is that the leaves have to be processed immediately for optimal product recovery. Also, Agrobacterium infiltration entails the presence of a large amount of bacterial cells, introducing the risk of bacterial endotoxin contamination. However, innovative, cost-effective purification systems, like the tobamovirus–protein A fusion, are being developed (Werner et al., 2006). Taken all together, the tobacco leaf-based expression system, both stable as well as transient, seems very promising.

In seed antibody expression

Seeds can be considered as a natural protein production factory and storage house. In addition to the merits of in planta expression, in seed expression enables oral delivery, long-term storage, and ease of handling and transport without cold chain maintenance (Floss et al., 2007, Khan et al., 2012). Seeds can be used directly for passive mucosal immunization, which is particularly advantageous for animal diseases (Floss et al., 2007, Zimmermann et al., 2009), and also for human application. Alternatively, for parenteral application, the high protein concentration in desiccated seeds facilitates the downstream processing. Different full-length antibody and antibody formats, like ScFv, ScFv-Fc, VHs and VHH-Fc, have been expressed effectively in seeds of both monocot and dicot plants (De Wilde et al., 2013, Khan et al., 2012, Loos et al., 2011b, Van Droogenbroeck et al., 2009, Virdi et al., 2013).

Although the use of the constitutive Cauliflower mosaic virus (CaMV) 3SS promoter (P3SS) to drive transgene expression can yield high amounts of antibodies in various plant tissues, its strength in controlling expression in seeds is poor, and a seed-specific promoter, like the β-phaseolin promoter leading to high accumulation is required (De Jaeger et al., 2002, De Wilde et al., 2013, Van Droogenbroeck et al., 2007). It is reasoned that the seed-specific promoters are usually active during the seed filling stage (development and maturity), while the CaMV P3SS ceases expression after the initial stages, explaining the lower accumulation (Chen et al., 1986, Petruccelli et al., 2006). Besides the β-phaseolin promoter (De Jaeger et al., 2002, De Wilde et al., 2013), several other seed-specific promoters, like USP (unknown seed promoter) (Zimmermann et al., 2009), glutelin-1 (gt-1) promoter (Ramessar et al., 2008), legumin A promoter (Perrin et al., 2000), and maize ubiquitin promoter (Christensen and Quail, 1996), have been evaluated for the expression of antibodies and other therapeutic proteins in plants (Khan et al., 2012).

Overall it has been seen that promoters specific for endosperm, in case of the monocot seeds, and those specific for cotyledons (i.e. the embryo), in case of dicot seeds, generally lead to high expression (Khan et al., 2012). Both these respective compartments are the protein sinks of the seed. Although compartment-specific promoters play a big role in the level of accumulation, they are not the sole factor (Drakakaki et al., 2006, Streetfield, 2007), since subcellular trafficking and accumulation play an equally important role. The endomembrane system of seeds differs from that of the other vegetative tissues. Seed cells have specialized subcellular structures called protein bodies (PBs), protein storage vacuoles (PSVs), starch granules and oil bodies. Some of these organelles, like PSVs and PBs, allow stable storage of antibodies, influencing their stability, accumulation and thus overall yield (Khan et al., 2012, Stöger et al., 2000). However, targeting the recombinant protein to a particular subcellular compartment can be difficult, since trafficking of native storage proteins is very complex and depends on the storage protein and the plant species (Khan et al., 2012). For instance, globulins and albumins are predominant storage proteins of many seeds, which usually reach the end destination of PSVs via the Golgi apparatus via dense vesicles, however in pumpkin seeds these proteins reach PSVs by omitting the Golgi apparatus, but instead traffic through precursor-accumulating vesicles (Hara-Nishimura et al., 1998, Hohl et al., 1996). Despite these complications in targeting recombinant proteins in seeds, the use of signal peptides of endogenous seed proteins and of the KDEL endoplasmatic reticulum (ER) retention signal, has enabled the successful expression and stable accumulation of several recombinant proteins in seed storage organelles (De Jaeger et al., 2013).
Secreting antibodies (by addition of an N-terminal signal peptide), and retaining them within the endomembrane system (with a C-terminal KDE/SEKDEL peptide) in general is a preferred strategy for seed antibody production. The specific retention also enables posttranslational modification like addition of glycans (Loos et al., 2011a). The glycosylation of proteins up to the ER is similar in yeast, plants and animals (Berends et al., 2009). Thus, the addition of plant-specific glycans is avoided by retention in the ER, especially in cases where glycosylation is important for efficacy in case of passive immunization therapy or intravenous administration.

It has been suggested that the bio-encapsulation in endomembranes protects proteins from the harsh gastric environment after oral delivery of crude or semi-processed seeds. When chickens were forced fed with pea seeds bearing anti-Eimeria ScFv, they were protected from subsequent coccidiosis, while purified antibodies (produced from tobacco leaves) failed to provide similar protection (Zimmermann et al., 2009).

### Conclusion

Despite the several advantages of plants as antibody expression systems, there are only a few antibodies that have made it through the clinical phase, such as the tobacco-produced CaroRx™ (Obembe et al., 2011). One of the main reasons for this delay in bringing plant-made therapeutic antibodies to the market could be the regulatory issues associated with genetically modified (GM) plants and the already established mammalian systems for monoclonal antibody production (Paul and Ma, 2011). Contamination of the environment with GM plants can be a serious issue. However, things are now changing mostly due to the development of expression technologies that have enabled transformation of different plant species, some of which can grow fast and produce large amounts of biomass in a short time. Moreover, the expression vectors have been improved, which enables high levels of antibody production from a reasonably small plant biomass (Paul and Ma, 2011, Whaley et al., 2011, Xu et al., 2012). This means that a substantial amount of antibody can be harvested from a limited number of plants that can be grown in contained glass facilities rather than on large open fields as initially anticipated. There is hope that in the near future the full potential of plants as a cost-effective platform would be realized.

So is plant expression the next disruptive technology for antibody production? In our opinion, perhaps not. It’s rather a niche technology. This is because the mammalian antibody expression system is well established, these antibodies are in clinics now and have thus gone through the cGMP production and drug regulation. Production of these clinically used antibodies in a new production platform as a ‘biosimilar’ is perhaps not worthwhile, given that it will have to go through regulatory compliance and although production costs might be lower than that of the mammalian expression system, it is uncertain if the market price of plant-made biosimilar antibodies would be significantly cheaper. However, the advantage of plants not harboring mammalian pathogens needs to be considered. One of the core strength of the plant expression system would be the production of ‘biobetters’, i.e. therapeutics with enhanced features attributed to enhanced efficacy, which is a notion shared by many leading experts in the field of plant molecular farming (Plant Based Vaccine, Antibodies and Biologies Conference 2013). Plant-made biobetter antibodies can be produced by banking on the strength of plant expression systems, particularly the rapid antibody production via automated cGMP transient tobacco expression systems, the homogenous glyco-engineered antibody production, the production of complex IgG and IgM antibodies, which can enable effective delivery via the oral route (Virdi et al., 2013). Thus, the plant expression technology is not a disruptive but a niche technology, which excels in the production of certain antibodies far beyond the conventional production platforms.

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