



# Current state-of-the-art in plant-based antibody production systems

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Received: 15 November 2018 / Accepted: 18 January 2019 / Published online: 25 January 2019  
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**Abstract** Monoclonal antibodies represent the major class of biopharmaceutical products (for therapeutics and diagnostics) with an increasing demand that reaches several tons per year worldwide. Traditional large-scale manufacturing processes are based on stirred tank bioreactors for the growth of Chinese Hamster Ovary cells (CHO) which requires high initial investments and production costs. Therefore, there is an urgent need for alternative production platforms that can at least act as a complement to the over-exploited mammalian fermentation systems. In this perspective, the use of plants for the large-scale production of biopharmaceuticals (‘Molecular farming’) represents an interesting and mature technology that has already proved its benefits in terms of safety, scalability, rapidity and reduced manufacturing costs. Here we discuss the recent advances in the production of monoclonal antibodies (mAbs) in plant-based platforms such as transgenic plants, tissue and cell cultures and transient expression systems.

**Keywords** Agroinfiltration · Glycosylation · mAb · Molecular farming · Plant expression systems · Transient expression

## Introduction

Monoclonal antibodies (mAbs) are useful tools in medicine, biology and biochemistry due to their binding specificity to different molecular targets and their stability both *in vivo* and *in vitro*. They represent the most successful biopharmaceuticals on the market with more than 50 mAbs currently approved and sales expected to increase to \$125 billion by 2020 (Shukla et al. 2017; Ecker et al. 2015). Mammalian cell cultures mainly based on Chinese Hamster Ovary cells (CHO) are still the favored system for the production of commercial mAbs, even if the increasing demand (also linked to the growing market of follow-on biologics -biosimilars-) is promoting the development of alternative expression platforms. Indeed, the facilities necessary for large-scale production in mammalian cells require high initial investments and their operating and maintenance costs are high (Ecker et al. 2015). Among alternative expression systems, plants represent promising bioreactors for the large-scale production of recombinant proteins and antibodies. They offer an attractive expression platform with several advantages such as the absence of potential human pathogens, possibility to engineer a tailored antibody glycosylation profile, possibility to scale-up production by simply increasing the number of plants and competitiveness of manufacturing costs. Nevertheless, expression of recombinant proteins in plants still presents several bottlenecks mainly related to

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purification and downstream processing as well as to regulatory issues (Fischer et al. 2015; Drake et al. 2017).

The estimated costs of mAb production in transgenic plants are calculated at about 100 €/g (Buyel et al. 2017) which are comparable to the average costs of 50–100 €/g reported in CHO cells (Lim et al. 2010; Kelley 2009). To strengthen the potentiality of this alternative production system a recent milestone was achieved by the private company Protalix Biotherapeutics Inc. (Israel) that developed a biopharmaceutical drug in carrot cells to treat a rare human metabolic disease (Gaucher's disease). ELELYSO™, the commercial name of the glucocerebrosidase enzyme used in replacement therapy, was the first plant-cell drug to receive the human use approval from FDA in 2012 (Mor 2015). This success story gave the impulse to other companies to invest in plant-based manufacturing facilities. For example, Medicago Inc. (Canada and US-based biopharmaceutical company) developed a quadrivalent seasonal influenza vaccine that has recently completed Phase III clinical trials (Efficacy, Safety, and Immunogenicity of a Plant-Derived Quadrivalent Virus-Like Particles Influenza Vaccine in Adults, <https://clinicaltrials.gov/ct2/show/NCT03301051?term=medicago&rank=2>) (Pillet et al. 2016). Until today, a wide range of recombinant proteins such as antibodies, enzymes and vaccines have been produced using plant-based expression systems and demonstrated efficacy and safety in pre-clinical as well as clinical studies (Yao et al. 2015). The results of some clinical evaluations are detailed in Table 1. In the next paragraphs, we will illustrate the major differences between glycoproteins produced in plants or mammalian cells and the most recent technologies used to efficiently produce mAbs in different systems such as whole plants, cell suspensions, hairy root cultures and microalgae.

### Plant-made versus mammalian cells derived glycoproteins

Post-translational modifications of proteins occurring in plant cells are essentially similar to those in animal cells and the correct assembly of complex molecules, such as antibodies, are assisted by chaperones that mediate the folding and formation of disulfide bonds while the addition of *N*-glycans is performed by

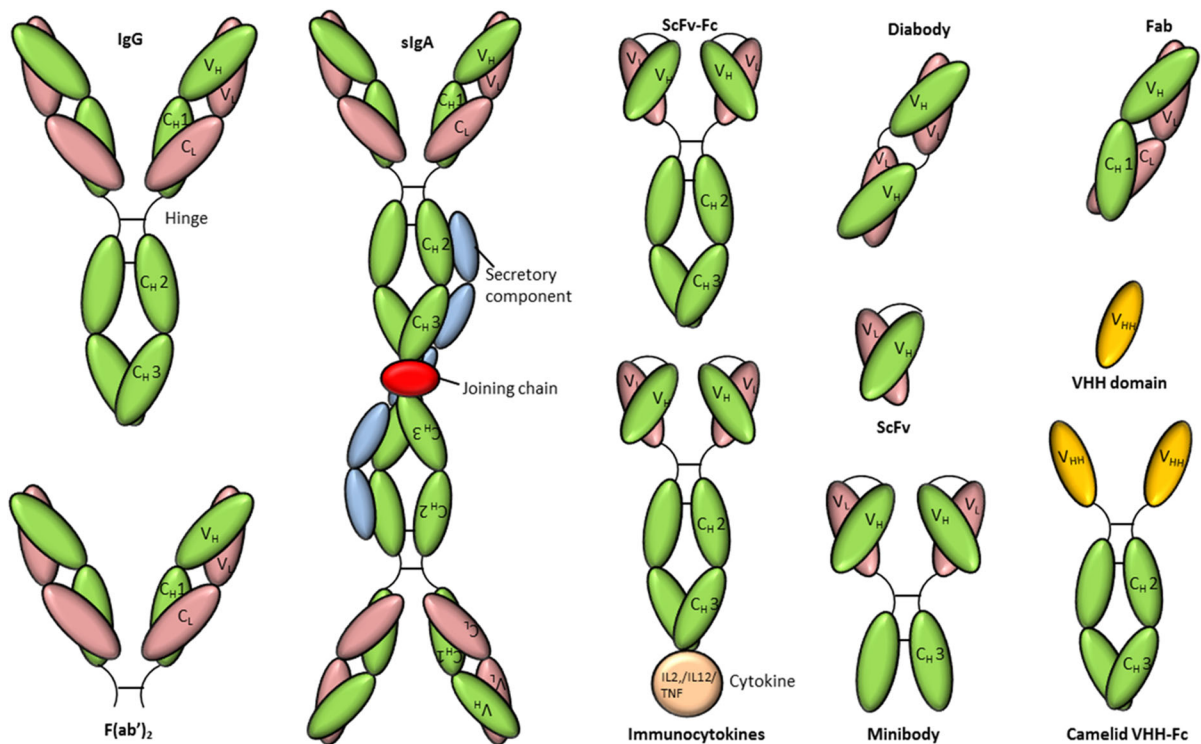
specific cellular glycosyltransferases. The *N*-glycans are those accounting for the most significant differences between plant-derived glycoproteins and their mammalian counterpart and are considered to be a major issue for the use of plant-derived pharmaceuticals in human therapy due to possible immunogenic reactions (Jin et al. 2008). In fact, while core *N*-glycans are similar in plants and mammals, complex *N*-glycans show substantial differences with sialic acid and  $\beta$  1,4-linked galactose residues typical of animal cells and  $\beta$ 1,2-xylose and  $\alpha$ 1,3-fucose of plants (Bosch et al. 2013). Potential immunogenicity of plant-typical sugar residues is still a controversial issue and until now clinical trials performed with plant-derived glycoproteins have never evidenced adverse effects on humans (Tusé et al. 2015). However, to overcome possible biosafety regulatory constraints this issue has been successfully addressed by 'optimizing' the *N*-glycosylation profile of plant-derived molecules by using different strategies such as the inactivation of endogenous plant-specific glycosyltransferases and/or their complementation by heterologous human glycosyltransferases (Steinkellner and Castilho 2015; Montero-Morales and Steinkellner 2018). For example, fucose and xylose-free glycoproteins were successfully obtained in whole plants or plant cell lines either using RNA interference or recent genome editing approaches (Jansing et al. 2018; Hanania et al. 2017). In many cases, mAbs produced in glyco-engineered plant systems have shown far better biological activity compared to those obtained in animal cells showing enhanced antibody-dependent cellular cytotoxicity (ADCC) and Fc- $\gamma$  receptor binding (Marusic et al. 2018; Qiu et al. 2014). Overall, recent advances in plant glyco-engineering allowed the production of 'human-like' recombinant glycoproteins with a highly homogeneous glycosylation pattern and plant expression systems can be considered versatile platforms for the manufacturing of mAbs with improved characteristics ('glycobetters').

### Production of mAbs in whole plants

The first pioneering study on the production of a full-size IgG in plants dates back to almost 30 years ago (Hiatt et al. 1989). Since then different antibody formats have been successfully expressed in plants such as secretory IgA, Fab fragments, single-chain

**Table 1** Examples of plant derived biopharmaceuticals (antibodies, vaccine antigens and enzymes) included in clinical trials or approved for therapeutic use

Product	Disease	Plant/expression system	Clinical trial stage	Company/Consortium	URL	References
<b>Antibodies</b>						
Chimeric mAb (CaroRX)	Dental caries	Tobacco/stable transformation	Phase 2	Planet Biotechnology	<a href="http://www.planetbiotechnology.com">http://www.planetbiotechnology.com</a>	(Weintraub et al. 2005)
Idiotypic IgG Based vaccine	Non-Hodgkin's lymphoma	<i>N. benthamiana</i> / Agroinfiltration	Phase 1	Icon Genetics		(Tusé et al. 2015)
Anti-HIV IgG	Prevention of HIV infection	Tobacco/stable transformation	Phase 1	Pharma-Planta Consortium	<a href="http://www.pharma-planta.net">http://www.pharma-planta.net</a>	(Ma et al. 2015)
Anti-Ebola IgG cocktail (ZMApp)	Treatment of Ebola virus infection	<i>N. benthamiana</i> / Agroinfiltration	Phase 2/3	Mapp Biopharmaceutical	<a href="http://mappbio.com">http://mappbio.com</a>	(PREVAIL II Writing Group et al. 2016)
IgG (ICAM1)	Common cold	Tobacco/stable transformation	Phase 1	Planet Biotechnology	<a href="http://www.planetbiotechnology.com">http://www.planetbiotechnology.com</a>	–
Radiolabeled anti-Ep-CAM IgG	Cancer treatment	Maize/stable transformation	Phase 2	NeoRx Corporation	–	(Gavilondo and Larrick 2000)
<b>Vaccine antigens</b>						
VLP-based Vaccine	Seasonal flu	<i>N. benthamiana</i> / Agroinfiltration	Phase 3	Medicago	<a href="http://medicago.com">http://medicago.com</a>	(Pillet et al. 2016)
VLP-based vaccine (H5N1)	Pandemic flu	<i>N. benthamiana</i> / Agroinfiltration	Phase 2	Medicago	<a href="http://medicago.com">http://medicago.com</a>	(Hendin et al. 2017)
<b>Enzymes</b>						
Glucocerebrosidase enzyme (ELELYSO)	Therapy of Gaucher's disease	Carrot/cell suspension culture	FDA Approved	Protalix Biotherapeutics	<a href="http://www.protalix.com">http://www.protalix.com</a>	(Mor 2015)
Alpha-galactosidase-A (Fabrazyme)	Therapy of Fabry disease	Tobacco/cell suspension culture	Phase 2	Protalix Biotherapeutics	<a href="http://www.protalix.com">http://www.protalix.com</a>	(Kizhner et al. 2015)
Alpha-galactosidase-A (moss-aGal)	Therapy of Fabry disease	Moss cultures	Phase 1	Greenovation Biopharmaceuticals	<a href="http://www.greenovation.com">http://www.greenovation.com</a>	(Shen et al. 2016)
Human deoxyribonuclease I (Aldomase alfa)	Treatment of cystic fibrosis	Tobacco/cell suspension culture	Phase 2	Protalix Biotherapeutics	<a href="http://www.protalix.com">http://www.protalix.com</a>	–



**Fig. 1** Examples of antibody molecules produced in plants

antibody fragments (scFvs), minibodies, single variable domains, antibody fusion proteins (immunocytokines), scFv-Fc antibodies and camelid heavy-chain antibodies (Fig. 1). These molecules were expressed in plants with different aims such as to confer virus resistance, to modulate plant cell processes but also for the production of valuable biopharmaceuticals including therapeutic mAbs (Lomonosoff and D'Aoust 2016).

Production of antibodies in whole plants was obtained using two different expression strategies based either on the stable transformation of the nuclear genome or on transient expression systems exploiting viral or *Agrobacterium tumefaciens* transfer DNA (T-DNA) expression vectors (Sheshukova et al. 2016). Among the bottlenecks of producing pharmaceutical proteins in whole plants, there is the necessity to consider the environmental impact and regulatory compliances, which often require growing plants in controlled conditions (contained greenhouses). This can be a limitation for the cost-effectiveness of this production platform since it greatly reduces the scaling-up potential compared to growing in the open

field. Conversely, in the case of transient expression systems that generally provide much higher yields and rapidity of recombinant protein accumulation, the production in contained greenhouses still allows an efficient scale-up and cost competitiveness of the manufacturing process. In the next paragraphs, the production of antibodies using either transgenic plants or transient expression systems will be illustrated.

#### Transgenic plants

Plants producing whole mAbs were traditionally obtained by cross-pollinating two transgenic lines separately transformed with the antibody heavy (HC) or light chain (LC) genes. This strategy was generally time-consuming but allowed the production of correctly assembled IgG molecules and more complex immunoglobulins such as secretory IgAs (Hiatt et al. 1989; Ma et al. 1995). More efficient and rapid strategies are now available based on the use of binary vectors containing both HC and LC coding sequences in the same T-DNA that allow to generate transgenic plants expressing complete IgGs in a single

transformation event (De Muynck et al. 2010). Several plant species can be efficiently engineered for the production of mAbs such as *Nicotiana benthamiana* (a wild relative species of tobacco), *Arabidopsis thaliana*, lettuce, potato and maize, but most antibodies reported in literature have been expressed in transgenic tobacco (*N. tabacum*) generally reaching levels of several milligrams per kg (Yusibov et al. 2016). Interesting results have also been obtained in the small aquatic plant *Lemna minor* (duckweed) in which a glycoengineered version of a mAb against human CD30 was obtained (Cox et al. 2006). The first antibody in Europe entering clinical trials was the 2G12 mAb produced in transgenic tobacco plants to be used as a microbicide against HIV infections (Ma et al. 2015). Other examples of antibodies that reached Phase II in the US were the CaroRx<sup>TM</sup> (secretory IgA) for the treatment of dental caries also obtained in tobacco (Weintraub et al. 2005) and Avicidin, an IgG against the epithelial cell adhesion molecule (EpCAM) for the treatment of refractive cancers (colon, lung and prostate) that was produced in maize (Gavilondo and Larrick 2000). Production and accumulation of recombinant proteins in seeds, a natural plant storage compartment, is an interesting alternative strategy that has several advantages such as the reduction of typical plant contaminants normally present in the leaves (e.g. alkaloids, phenolic compounds etc.) and the possibility to enhance long-term protein stability without the need of storing the plant material at low temperatures (Boothe et al. 2010). An interesting example of this strategy was the expression of the neutralizing anti-HIV-1 2G12 antibody in seeds of transgenic maize which allowed to obtain a yield of 75 mg per kg of dry seed weight (Rademacher et al. 2008).

#### Transient expression systems based on agroinfiltration technology

Transient or epichromosomal transformation differs from stable transformation in that the exogenous sequence is not inherited by the progeny thus reducing the risk of environmental biosafety issues linked to the dissemination of the transgene through seeds or pollen. This approach generally provides high protein yields in a very short period of time (few days to weeks) which is not achievable via stable transformation (Komarova et al. 2010). Transient expression can

be obtained using either vectors or plasmids containing T-DNA gene cassettes derived from *A. tumefaciens* bearing a strong constitutive promoter or vectors carrying appropriately modified genomic sequences of plant viruses inserted in T-DNA cassettes (viral vectors) (Kopertekh and Schiemann 2017).

The Agrobacterium-mediated expression systems based on T-DNA gene cassettes are typically made up of vectors carrying multiple cloning sites, a strong constitutive promoter, selectable marker genes and plasmid replication components for *E. coli* and *A. tumefaciens*. These vectors exploit the ability of *A. tumefaciens* to transfer the T-DNA bearing the gene of interest in the plant cells. This methodology, known as agroinfiltration, is based on the permeation of the agrobacterium containing solution in the intercellular spaces of the leaves by using a needleless syringe or through vacuum (Chen and Lai 2015). These methods provide synchronous gene expression because agrobacterium is known to simultaneously infect almost 100% of the cells in the infiltrated leaves. Transient expression based on leaf agroinfiltration was successfully used to produce fully functional mAbs in different plant species such as *N. benthamiana*, tobacco, lettuce and tomato (De Muynck et al. 2010). Moreover, to enhance antibody accumulation in plant tissues, the co-expression of plant virus gene-silencing suppressor proteins (e.g. P19 from Artichoke mottled crinckle virus -AMCV-) proved to be an effective strategy increasing protein expression levels up to tenfold (Circelli et al. 2010). A further advantage of this technology is also the rapid scalability of the process which gives the possibility to handle large numbers of plants by using automated vacuum-agroinfiltration systems on large-scale under Good Manufacturing Practice (GMP) Regulations (Chen et al. 2013).

Most recent and efficient transient expression systems, also based on T-DNA gene cassettes, rely on the use of virus-based expression vectors delivered in plant tissues by agroinfiltration. In the first generation of viral vectors the gene of interest is inserted as an additional Open Reading Frame in the functional genome of the plant virus (“full virus” strategy) (Lico et al. 2008). To overcome the limitations of this strategy, mostly concerning the size of the foreign gene tolerated by the viral genome, a second generation of virus expression vectors was engineered. These include only the viral elements (strong



promoters, terminators, enhancers, silencing suppressor genes) required for the expression of the foreign gene and are referred to as the “deconstructed virus” strategy (Gleba et al. 2007). This allowed developing a technique called ‘Magniffection’ that drastically facilitated large-scale manufacturing processes and most importantly enhanced protein yields up to 100 times. The expression platform commercially known as ‘MagnICON’ proved to be very effective for the high-yield production of mAbs in *N. benthamiana* (Giritch et al. 2006; Klimyuk et al. 2014). A successful example of the exploitation of this technology is the Phase 1 clinical study conducted on chimeric antibodies for the treatment of B cell follicular lymphoma essentially by using these molecules as individualized idio-type vaccines created from patient’s own cancer cells. The rapid and high-level expression offered by plants permitted to proceed from biopsy to the individualized vaccine in less than 12 weeks (Tusé et al. 2015). Another advanced application of the ‘Magniffection’ technology led to the production of a cocktail of three neutralizing mAbs (ZMapp) developed for the treatment of Ebola virus infection in glycoengineered *N. benthamiana* plants with knocked-down plant-specific glycosyl transferases ( $\beta$ 1,2-xylosyltransferase; and  $\alpha$ 1,3-fucosyltransferase) (Qiu et al. 2014; Strasser et al. 2008). The ZMapp cocktail was administered to 71 patients during the 2015 Ebola outbreak in West Africa under compassionate-use protocols. In this randomized, controlled trial the estimated effect of ZMapp appeared to be beneficial although the result did not meet the statistical threshold for efficacy (PREVAIL II Writing Group et al. 2016). Recently, the ‘Magniffection’ technology allowed to reach the highest results in terms of antibody production yield in whole plants with levels of 2 g per kg of fresh weight (Zischewski et al. 2016).

Another efficient viral-derived expression system is based on the Cowpea mosaic virus (CPMV). This virus has a bipartite RNA which has been used to develop deconstructed expression vectors for the production of antibodies (Sainsbury et al. 2010). A recent development of this technology is the ‘hyper-translatable’ Cowpea mosaic virus-based protein expression system (‘CPMV-HT’) that allows the expression of multimeric proteins, encoded by genes in a single T-DNA construct, using the agroinfiltration system (Saxena et al. 2016). This viral vector was

successfully used to accumulate high levels of the anti-HIV mAb 2G12 in *N. benthamiana* plants and the purified antibody showed to be functionally equivalent to the same molecule produced in mammalian cells (Sainsbury et al. 2010). In recent years, several biotechnology companies operating mainly in the United States, have built semi-automated systems for the large-scale production of plant-derived bio-pharmaceuticals using agroinfiltration-based transient expression platforms (Holtz et al. 2015).

## Production in plant cells and tissue cultures

Plant tissues and plant cell-based platforms represent a valid system for the production of heterologous proteins thanks to the possibility of producing in sterile contained conditions with low risks of contamination by ‘human pathogens’ (Schillberg et al. 2013). Furthermore, transformed plant cells and organs can be propagated indefinitely and the protein of interest can be secreted into the culture medium facilitating the product recovery and purification (Magy et al. 2014). The following paragraphs summarize the most promising plant cell and tissue culture systems used for the production of biopharmaceuticals as well as future perspectives and challenges.

### Plant cell suspension cultures

For more than two decades undifferentiated plant cell suspension cultures have been used for the production of heterologous proteins. Plant cell suspensions are usually prepared from callus tissue in shaker flasks or fermenters to form single cells and small aggregates. The most used plant cell lines are derived from *N. tabacum* (such as BY2 or NT1 cells) while some others originate from edible crop species such as rice, soybean, carrot and tomato (Santos et al. 2016; Xu and Zhang 2014). Cultured plant cells require simple nutrients to grow and the purification and downstream process is easy because they generally allow the accumulation of the recombinant protein in the culture medium even if size and composition of the foreign protein may influence its secretion capacity (Schillberg et al. 2013). A weakness of this system is the low yield of secreted protein (in the range of milligrams per liter of culture) that is also influenced by the proteolytic activity in the culture medium (Magy et al.

2014). The first human protein expressed in *N. tabacum* cell suspension cultures dates back to 1990s when the correctly processed human serum albumin was obtained (Sijmons et al. 1990) but several other proteins such as cytokines, hormones, vaccine antigens and antibodies have been produced since then (Schillberg et al. 2013). A striking successful example of the exploitation of plant cell-based bioreactors was the production of the drug ELELYSO™ to treat Gaucher's Disease in carrot cells by using a cost-effective plant cell culture platform called ProCellEx™ that significantly reduced the manufacturing costs of this drug (Tekoah et al. 2015). The same culture platform was recently used to produce the enzyme alpha-galactosidase-A ('Fabrazyme') in tobacco cells for the treatment of the rare genetic Fabry disease (Kizhner et al. 2015).

Antibodies remain the most expressed proteins because of their diagnostic and pharmaceutical value. An example is the tumour targeting monoclonal antibody M12 that was obtained in BY-2 cells using a 200 litres disposable bioreactor with yields of 20 mg l<sup>-1</sup> (Raven et al. 2015) while the optimization of the culture medium allowed raising the yield of this antibody to 100 mg l<sup>-1</sup> (Vasilev et al. 2013). Among the advantages of using cell suspension cultures, there is also the possibility to optimize the glycosylation profile of proteins by engineering the glycan metabolism of plant cells. An example was the establishment of stably engineered BY-2 cell lines that co-expressed the human  $\beta$ -1,4-galactosyltransferase enzyme or in which the  $\beta$ (1,2)-xylosyltransferase and the  $\alpha$ (1,3)-fucosyltransferase genes were inactivated. These lines were successfully used to produce antibodies with a human-like glycosylation profile (Mercx et al. 2017; Navarre et al. 2017). Recently, a novel expression system was developed based on plant cell suspension packs (or cell 'cookies'). These cell 'cookies' are directly transformed with a recombinant *Agrobacterium* bearing the gene of interest and incubated for a few days until the desired level of protein has been reached (Rademacher 2013).

### Hairy root cultures

Among cultured plant tissues, hairy roots (HR) represent an interesting novel platform for the production of heterologous proteins. HR are neoplastic tissues that originate upon infection of monocot or

eudicot plant species with *Agrobacterium rhizogenes* (now revised as *Rhizobium rhizogenes*) a gram-negative soil bacterium of the family Rhizobiaceae that is naturally able to introduce into the genome of the infected plant a T-DNA segment from its root-inducing (Ri) plasmid (White et al. 1985). This T-DNA carries a set of oncogenes that promote the formation of proliferating HR that can be stably grown in sterile cultures for several years. Since decades HR have found numerous applications in the production of bioactive secondary metabolites but just recently they have been considered as possible bioreactors for the manufacturing of biopharmaceutical proteins (Mehrotra et al. 2015). HR offer several advantages such as genetic stability, growth in sterile contained conditions, fast biomass accumulation and the possibility to secrete the heterologous proteins in the culture medium. On the other hand, the low protein yields (in the range of mg per liter of culture) and the difficulties in setting up large-scale production in bioreactors represent the major challenges for the future exploitation of this plant expression platform. Yet there are many examples in the literature of different classes of heterologous proteins that have been successfully produced in HR such as enzymes, vaccine antigens, growth factors, interleukins and antibodies (Xu et al. 2012). The first biopharmaceutical protein to be expressed in HR was the Anti-*Streptococcus mutans* mAb Guy's 13 that was successfully secreted in the culture medium (Wongsamuth and Doran 1997). A recent example of a mAb expressed in HR, is the anti-vitronectin mAb M12 tumour-targeting antibody (Häkkinen et al. 2014). In this work, an optimised induction protocol for the cultivation of tobacco HR secreting the mAb M12 in the culture medium enhanced antibody yield by 30-fold and about 57% of the antibody produced was secreted in the medium. Characterization of the purified antibody showed that it possessed a typical plant glycosylation pattern, which still represents a major issue for plant-derived antibodies to be used in human therapy. In a recent approach, tumour-targeting anti-CD20 and anti-tenascin C antibodies and immunocytokines were expressed in glyco-engineered *N. benthamiana* HR cultures in which  $\beta$ (1,2)-xylosyltransferase and the  $\alpha$ (1,3)-fucosyltransferase genes were silenced (Lonoce et al. 2016, 2018). These examples demonstrated that it is possible to produce

fully functional anticancer antibodies with a human compatible glycosylation profile in HR.

An advantage of hairy roots compared to expression platforms based on whole plants is the possibility of secreting the recombinant proteins in the culture medium. It was demonstrated that antibody purification from HR culture medium could be carried out using a simple two-step procedure composed by a first clarification of the medium by filtration and an affinity chromatography step using Protein A resin. In the case of anti-CD20 antibodies, Lonoce and colleagues reported a 60% protein recovery with final yields of 20 mg l<sup>-1</sup> of purified product (Lonoce et al. 2018).

### Moss cultures

Moss cultures have received increasing interest as a novel system for the production of biopharmaceutical proteins in bioreactors (Decker et al. 2014). Moss cells have the ability to photosynthesize and can be grown in self-contained systems like bioreactors. Furthermore, recombinant proteins can be secreted into the culture medium simplifying the purification process. The moss *Physcomitrella patens* has been widely used for the production of different classes of biopharmaceuticals mostly due to its almost complete genetic characterization which makes it an ideal organism for precise gene targeting via homologous recombination. This type of genetic manipulation allowed, for example, glyco-engineering approaches for the elimination of plant-typical immunogenic residues leading to the production of safe therapeutic proteins. This approach was used for the production of the glycoengineered version of the tumour-targeting lewis Y-specific mAb MB314 which proved to be far more efficient in ADCC than the CHO-derived antibody (Kircheis et al. 2012). A recent achievement was made by the Greenovation Biopharmaceuticals company that developed a moss culture-based production platform (Bryo-Technology) allowing high-quality proteins to be produced on large-scale under GMP conditions using disposable wave reactors (Reski et al. 2015; Shen et al. 2016).

### Microalgae cultures

Plants are not the only photosynthetic organisms that can be exploited for the production of recombinant proteins. Microalgae, for example, are unicellular species with a size range of a few micrometers (µm) to 100 µm that can be used as biofactories of different molecules (Potvin and Zhang 2010). The major advantage of algae is their high growth rate and rapid accumulation of biomass, ease of cultivation in contained bioreactors and their ability to carry out post-translational modifications of proteins such as glycosylation. The major drawback is that the yields of pharmaceutical proteins obtained with this system are still very low compared to transient expression systems using whole plants and glycoengineering attempts to produce proteins with human-like glycosylation profiles have not been successful so far (Mathieu-Rivet et al. 2014; Vanier et al. 2017). Nevertheless, there are many examples in the scientific literature of the use of several species of microalgae (*Chlamydomonas reinhardtii*, *Phaeodactylum tricorutum*, *Dunaliella salina* and *Schizochytrium sp*) for the expression of different classes of pharmaceutical proteins such as vaccines, enzymes and antibodies (Doron et al. 2016). *Chlamydomonas reinhardtii* is certainly the most widely used green microalgae for recombinant protein production due to its low manufacturing costs and the ease in scaling up the production process. Furthermore, the genome of this species has been sequenced and different genetic tools are available for its transformation facilitating recombinant protein production (Shamriz and Ofoghi 2016). The first example of the expression of a recombinant antibody molecule in microalgae was obtained in the chloroplast of *C. reinhardtii* in which a functional single-chain antibody (HSV8-lsc) directed against glycoprotein D of the herpes simplex virus accumulated in a functional form (Mayfield et al. 2003). Recently, the diatom *P. tricorutum* was engineered to express a full mAb directed against the Hepatitis B virus surface antigen (HBsAg), either secreted or retained in the endoplasmic reticulum (Hempel and Maier 2012; Vanier et al. 2018). These examples show the potential of microalgae to produce fully assembled and functional antibodies, although the production yields are still far from being competitive with other plant systems.



### Conclusions and future scenarios

In this review, we highlighted the extremely diversified scenarios of biopharmaceuticals plant-based production platforms (Fig. 2). The different manufacturing strategies proved their advantages in the development of new drugs some of which were recently approved for human therapy. These success stories rely on peculiar features of plant-based platforms that in specific cases can outcompete with traditional animal-based systems. An example is the production of the seasonal influenza vaccine for which manufacturing promptness is an essential prerequisite to obtain an efficacious drug against the most diffuse viral isolates. For this reason plant transient expression systems based on agroinfiltration technology, thanks to their production flexibility and rapidity, proved to be particularly suitable for the preparation of such vaccines. Another example is the production of the

ZMapp antibody cocktail against Ebola. In this case, only a very flexible manufacturing system could have allowed the simultaneous production of three different viral neutralizing antibodies to be used in passive immunotherapy. Probably, the most unique feature of plant-based systems is the possibility of producing ‘glycobetter’ proteins. The commercial success of the glucocerebrosidase enzyme (ELELYSO™) to treat Gaucher’s disease relies mainly on its high mannose glycosylation profile that gives superior quality compared to the equivalent animal cell-derived drug and could only be obtained by using plant cells. Also in the case of immunotherapeutic antibodies, we have shown that the use of glyco-engineered plants allowed the production of tailor-made mAbs with enhanced biological activity compared to their mammalian derived counterparts. Finally, future scenarios envisage a dramatic escalation of the demand of certain biopharmaceuticals such as mAbs to treat common diseases




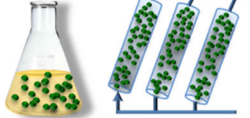
		Whole plants	Cell / Tissue cultures	Moss / microalgae cultures	
		<b>Transgenic plants</b> 	<b>Agroinfiltration</b> 		
Challenges	Advantages	<ul style="list-style-type: none"> <li>✓ Production at very large-scale (tons of recombinant protein)</li> <li>✓ Competitiveness of costs at large-scale</li> <li>✓ Possibility of accumulating the recombinant protein in seeds (long-term protein stability without the need of the cold chain)</li> </ul>	<ul style="list-style-type: none"> <li>✓ Rapid and flexible production platform</li> <li>✓ High protein expression yields</li> <li>✓ Scale-up with semi-automated systems in greenhouse</li> <li>✓ No environmental safety issues (production in greenhouse allows containment)</li> <li>✓ Use of glyco-engineered plants</li> </ul>	<ul style="list-style-type: none"> <li>✓ Growth in contained bioreactors (no contamination by pathogens)</li> <li>✓ Compliance with current regulatory standards</li> <li>✓ Easy downstream processing (protein secreted in the medium)</li> <li>✓ Drug produced in plant-cell cultures already on the market</li> <li>✓ Use of glyco-engineered cell/tissue lines</li> </ul>	<ul style="list-style-type: none"> <li>✓ Growth in contained bioreactors (no contamination by pathogens)</li> <li>✓ Compliance with current regulatory standards</li> <li>✓ Easy downstream processing (protein secreted in the medium)</li> <li>✓ Use of glyco-engineered moss lines</li> </ul>
	Challenges	<ul style="list-style-type: none"> <li>✗ Environmental containment issues concerning GM plants in the open field</li> <li>✗ Low protein expression yields</li> <li>✗ Downstream processing issues related to extraction of proteins from leaves</li> <li>✗ Regulatory hurdles</li> </ul>	<ul style="list-style-type: none"> <li>✗ Downstream processing issues related to extraction of proteins from leaves</li> <li>✗ Higher investment costs at large-scale production compared to transgenic plants</li> <li>✗ Removal of Agrobacterium endotoxins</li> <li>✗ Possible regulatory hurdles</li> </ul>	<ul style="list-style-type: none"> <li>✗ High investment costs required</li> <li>✗ Low protein expression yields</li> <li>✗ Difficulty in culture scale-up (especially for hairy roots)</li> </ul>	<ul style="list-style-type: none"> <li>✗ High investment costs required</li> <li>✗ Low expression yields are generally obtained</li> <li>✗ Culture scale-up is complex</li> <li>✗ Glycoengineering of microalgae is difficult</li> </ul>

Fig. 2 Advantages and challenges of the different biopharmaceuticals plant-based production platforms

(Alzheimer's disease, HIV, rheumatoid arthritis etc.) which will require production on a very large scale (hundreds of tons per year). If this would be the case, production using transgenic plants that can be easily and cost-effectively up-scaled by just increasing the cultivated area could be an ideal alternative to animal-based systems.

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