Engineering Plants for the Future: Farming with Value-Added Harvest

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Abstract Plants and their rich variety of natural compounds are used to maintain and to improve health since the earliest stages of civilization. Despite great advances in synthetic organic chemistry, one fourth of present-day drugs have still a botanical origin, and we are currently living a revival of interest in new pharmaceuticals from plant sources.

Modern biotechnology has defined the potential of plants to be systems able to manufacture not only molecules naturally occurring in plants but also newly engineered compounds, from small to complex protein molecules, which may originate even from non-plant sources. Among these compounds, pharmaceuticals

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such as vaccines, antibodies and other therapeutic or prophylactic entities can be listed. For this technology, the term plant molecular farming has been coined with reference to agricultural applications due to the use of crops as biofactories for the production of high-added value molecules. In this perspective, edible plants have also been thought as a tool to deliver by the oral route recombinant compounds of medical significance for new therapeutic strategies. Despite many hurdles in establishing regulatory paths for this "novel" biotechnology, plants as bioreactors deserve more attention when considering their intrinsic advantages, such as the quality and safety of the recombinant molecules that can be produced and their potential for large-scale and low-cost production, despite worrying issues (e.g. amplification and diffusion of transgenes) that are mainly addressed by regulations, if not already tackled by the plant-made products already commercialized. The huge benefits generated by these valuable products, synthesized through one of the safest, cheapest and most efficient method, speak for themselves.

Milestone for plant-based recombinant protein production for human health use was the approval in 2012 by the US Food and Drug Administration of plant-made taliglucerase alfa, a therapeutic enzyme for the treatment of Gaucher's disease, synthesized in carrot suspension cultures by Protalix BioTherapeutics.

In this review, we will go through the various approaches and results for plant-based production of proteins and recent progress in the development of plant-made pharmaceuticals (PMPs) for the prevention and treatment of human diseases. An analysis on acceptance of these products by public opinion is also tempted.

Keywords Plant molecular farming, Plant-derived antibodies, Plant-derived vaccines, Responsible research and innovation

1 Introduction

Plants have provided mankind with useful molecules for centuries. The idea to produce heterologous (exogenous, non-plant) protein in plants to get plant-made pharmaceuticals (PMPs) has been available and rapidly advanced, for just over 30 years (Ma et al. 2003). The first plant-made pharmaceutically relevant protein was the human growth hormone, expressed in transgenic tobacco in 1986 (Barta et al. 1986). Other human proteins have been produced in an increasing variety of plant species and of techniques. In 1989, the expression of the first antibody in tobacco (Hiatt et al. 1989) and, subsequently, the production of the first experimental plant-made vaccine (e.g. hepatitis B virus surface antigen) (Mason et al. 1992) clearly showed that plants had the capability to assemble functional glycoproteins with complex structure. The structural authenticity and preservation of function of plant-produced recombinant proteins was confirmed by the production of more complex antibody derivatives such as the secretory immunoglobulin A (Ma et al. 1995) and new immunogenic proteins (Haq et al. 1995). Also antigenic peptides, expressed as fusions to plant viral coat proteins, were made, and, in many cases, the assembled viral-like particles were demonstrated able to protect animals from the challenge

with the pathogen from which the epitope was derived (Rybicki 2014; Kushnir et al. 2012). At the same time, fusions with plant-derived proteins or only plant-typical sequences were found to improve the immunogenicity of antigens for vaccination against tumours (Massa et al. 2011, 2017).

The evolution of molecular techniques has led to the development of systems like the Gateway-mediated cloning (reviewed by Dafny-Yelin and Tzfira 2007), Golden Gate (Binder et al. 2014) and GoldenBraid (Sarrion-Perdigones et al. 2011) just to name a few, thanks to which even the assembly of multi-modular constructs for the expression of multiple proteins or enzymes in plants is now possible.

Beside proteins for pharmacological use, also plant-made industrial products (PMIPs) have been produced, such as enzymes, proteins for research use, nutritional supplements, polymers, etc. (Davies 2010; Tschofen et al. 2016).

Unlike mammalian cell-derived drugs, plant-derived antibodies, vaccines and other pharmacologically relevant proteins are particularly advantageous in that they are free from mammalian viral vectors and/or human pathogens. Advantages offered by plants may include also low cost of cultivation and high biomass production, relatively fast "gene-to-protein" time, low investment costs, good scalability, eukaryotic post-translational modifications and a high protein yield (Table 1).

The availability of effective technical means of expressing proteins in plants offers the prospect of using them as bioreactors for the production of cost-effective pharmaceuticals for both human and animal health sectors.

Over the past four decades, a wealth of literature has demonstrated that the production of proteins in plants for health applications is a promising approach in the area of biologics manufacturing. It is hoped that together to what has been defined in the MIT Technology Review "the biggest biotech discovery of the century", the CRISPR technology, new frontiers of biomedical discoveries, ranging from the pharmaceutical sector to the agro-industry, will be opened and ready for this "next-generation" plant-based medicine.

In its intent, the present review is thought to make the point on the evident opportunities that arise from the "Farming for Pharming" of biologics through plants. If properly addressed, this technology is destined to tackle human and animal diseases breaking the limits of current standard production technologies of biopharmaceuticals. This in turn will help reduce disparities in health rights and guarantee better health protection in the name of the guiding principle of reduction of costs.

2 The Strategies and the Technological Platforms of Plant Transformation

The diversity of plant molecular farming systems available reflects the wealth of possibilities offered by plants. Recombinant proteins have been produced in many different plant species wherein there is a choice of whole plants or various cell/tissue

Table 1 Comparison of expression hosts for the production of proteins of pharmacological interest (content partially sourced from Ma et al. 2003; Franconi et al. 2010; Loh et al. 2017)

Host	Expression level	Production time	Production cost	Scale-up capacity	Glycosilation pattern (vs human)	Risk of contamination
Bacteria	Medium/ high	Short	Low/ moderate	High	None	High: endotoxins
Yeast	Low/high	Medium	Medium	High	Incorrect: high mannosylation	Low
Insect cells	Low/high	Medium	High	Medium	Incorrect: high mannosylation	High: baculoviruses and mammalian viruses
Mammalian cells	Low/ medium	High	High	Very low	Correct	High: mammalian viruses, prions, oncogenic DNA
Animal	Medium/ high	Very high	High	Low	Correct	High: mammalian pathogens, prions, oncogenic DNA
Plant cell cultures	Medium/ high	Short	Low-moderate	High	Minor differences, possibility to modify	Very low
Plant organs (e.g. hairy roots)	Medium/ high	Medium/ short	Low-moderate	High	Minor differences, possibility to modify	Very low
Whole-plant transient (agro-infiltration)	Medium/ high	Medium	Low	High	Minor differences, possibility to modify	Very low
Whole-plant stable (nuclear/plastidial)	Medium/ high	Long	Low	High	Minor differences, possibility to modify	Very low

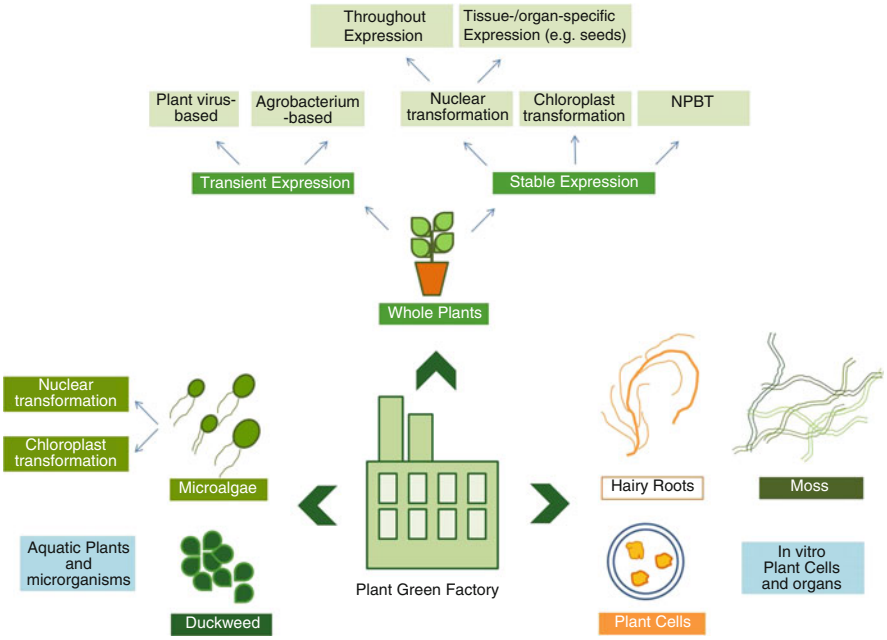

Fig. 1 The plant factory expression platforms (content partially sourced from Xu et al. 2012; NPBT: new plant breeding techniques)

culture formats (Fig. 1). Plants may be suitable for stable expression (including nuclear and plastid transformation in some species) and transient expression (which can be achieved using *Agrobacterium tumefaciens*, plant virus vectors or combinations of both). The various approaches for plant-based production of proteins are schematically illustrated in Fig. 2.

2.1 Stable Transformation

2.1.1 The "Whole-Plant" Approach

Stable nuclear and chloroplast transformations are the two approaches used to stably express heterologous (i.e. non plant) recombinant proteins in plants. *Agrobacterium*-mediated and particle bombardment-mediated stable transformation have a long history and are achieved by stable integration of DNA into a plant genome which results in the expression of transgenes (Tinland 1995).

In the field of whole-plant transformation, mainly tobacco and its relative *Nicotiana benthamiana*, which has a fast growth rate and an apparent defective RNA silencing system (Goodin et al. 2008), potato, tomato and lettuce are the main leafy crops used with the primary advantage of an abundant leaf biomass

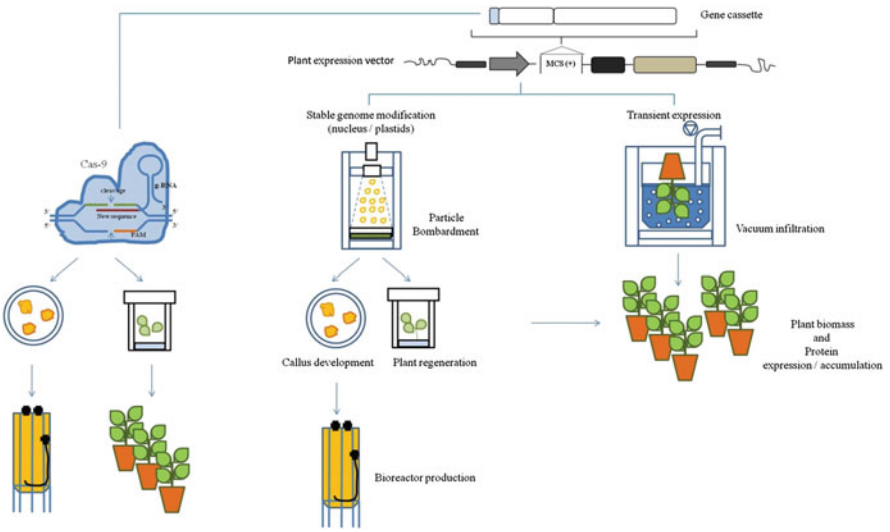

Fig. 2 Production of recombinant proteins in plants using stable or transient transformation methods (partially sourced from Loh et al. 2017)

accumulation accompanying the expression of the exogenous protein (Tremblay et al. 2010). Among cereals, maize, rice and barley (Nandi et al. 2005; Sabalza et al. 2013) have been tried along with soybean, among legumes. While the watery tissues of leaves impose to extract rapidly the target protein in order to minimize degradation, desiccated seeds allow protein to remain stable and are suitable for a possible oral administration.

Despite the large array of plant systems available and the great advantage of playing a seminal role in the development of "green" bioreactors, the "whole-plant" transgenic approach is labour-intensive and time-consuming due to the necessary steps for plant regeneration and selection, with an average lead time ranging from 12 to 18 months to engineer plants that often show non-competitive contents of the target protein (Gleba et al. 2005).

Stable introduction of target genes into chloroplast genome (transplastomics) allows for higher levels of expression if compared to nuclear transformation, due to the lack of gene silencing phenomena and high gene copy numbers. The technique is also advantageous since it prevents possible transgene escape (plastids are inherited through the maternal tissue in the majority of species) and absence of chloroplasts in pollen and improbability of their transfer, which quells environmental concerns (Meyers et al. 2010; Cardi et al. 2010). This method allows high concentration of recombinant protein (Oey et al. 2009). Nevertheless, it is technically difficult since several generations of plant regeneration are needed to select transformed plants with all chloroplasts bearing the gene of interest (homoplasmic state). In addition, this platform lacks most post-translational modifications being successful in a limited number of species.

Aquatic plants like duckweed (*Lemna minor*) and other non-vascular plants such as moss (*Physcomitrella patens*) have been considered as alternative expression platforms (Reski et al. 2015). Having properties in common with both terrestrial plants (in that they are differentiated whole plants) and cell suspension cultures (they can be grown in containment and in simple mediums), technologies applicable to aquatic plants and cell suspension cultures are similar with the difference being that aquatic plants require light, while cell suspension cultures are usually grown in the dark with the addition of a carbon source.

2.1.2 Cell Suspension Cultures

Removal of cell walls and gene transfer into the resulting protoplasts and suspension culture is an easier method in comparison to whole-plant genetic manipulation, since this might simplify purification and downstream processing (Santos et al. 2016). In addition, suspension culture allows homogeneity in cell proteins and sugars (*N*-glycans) due to the high uniformity of type and cell size (Lienard et al. 2007). Plant cells can be cultivated aseptically using classical fermentation technology, are easy to scale up and must comply with regulatory requirements similar to those established for consolidated systems based on both microbial and mammalian cells. In fact, plant suspension cultures, being made of single cells, allow comparisons with the mostly used mammalian cell production of recombinant pharmaceuticals, Chinese hamster ovary (CHO) cells (Table 2). Cell-specific production rates of 8 pg/cell/day have been reported for the monoclonal antibody M12 produced in tobacco BY-2 cells (Havenith et al. 2014) compared to typical production rates of 20–40 pg/cell/day for CHO cells (typically carrying thousands of gene copies), showing that the difference between these systems is less than an order of magnitude (Santos et al. 2016).

Tobacco cell lines BY-2 and NT-1 have been used to produce many recombinant proteins (Ullisch et al. 2012), but other platforms include carrot (*Daucus carota*) and rice (*Oryza sativa*) cell lines (Hellwig et al. 2004; Xu et al. 2012). Innovative methods have been also developed for the production of cell packs from suspension cultures to facilitate accumulation and purification of target proteins (Rademacher 2013).

Table 2 Differences in biopharmaceutical production between mammalian and plant cell cultures

Mammalian cell production	Plant cell production
High initial investment (>$250 million; e.g. expensive stainless steel bioreactors)	Low initial investment (<$250 million; e.g. inexpensive polyethylene bags)
Long timeline for capacity expansion	Rapid scale-up and capacity expansion
Growth and manufacturing under strict controlled environment	Growth and manufacturing at room temperature
Expensive maintenance	Less costly “hands-on” maintenance
Risk of human pathogen contamination	No risk of human pathogen contamination

It should be noticed that taliglucerase alfa (Elelyso®), the first plant-made licensed recombinant pharmaceutical protein, is produced by carrot cell suspension cultures (Shaaltiel et al. 2007) as well as the veterinary vaccine for poultry against Newcastle disease approved by the US Department of Agriculture that is produced in suspension-cultured tobacco cells.

Despite general concerns about a possible genetic instability, plant cell cultures are fundamentally a reliable system in which medium optimization, process engineering, experimental/process design and scale-up can be tuned on demand (reviewed in Santos et al. 2016).

2.1.3 Green Unicellular Microalgae

In the recent past, other systems have been devised, which can easily be contained, propagated and transformed to produce recombinant proteins. A protein expression system that is based on the unicellular green alga *Chlamydomonas reinhardtii* has been developed (Mayfield et al. 2003). This alga has nuclear, plastidial and mitochondrial genomes completely sequenced. In this system, chloroplast-targeted transgenes were used to express different recombinant, health-related proteins. Like bacteria, the chloroplast lacks the machinery to perform complex post-translational modifications such as glycosylation (the glycosylated proteins come from the endoplasmic reticulum), but, unlike *E. coli*, *Chlamydomonas* chloroplast allows the disulphide bond formation and is able to carry out some types of phosphorylation. Unlike higher plants, *C. reinhardtii* has a single chloroplast, with about 80 genome copies. Consequently, conversion of all copies of the chloroplast genome to recombinant homoplasmy is facilitated. Complex molecules such as fully functional antibodies, therapeutics (among which a candidate therapeutic vaccine against human papillomavirus-related tumours based on a soluble, immunogenic form of the E7 viral protein) and other biologics have been produced with various yields highlighting the potential of microalgae as alternative platforms for the production of biologics for human uses (Mayfield et al. 2003; Demurtas et al. 2013). This relatively novel platform offers advantages including short time from transformation to scaling-up, rapid growth (doubling time of few hours), ease of cultivation, safety (microalgae do not harbour human pathogens and many are generally regarded as safe (GRAS) organisms) facilitating production of biopharmaceuticals in GMP conditions and homogeneity of protein production. Technologies for molecular pharming in *C. reinhardtii* are still in the infancy, while efforts are made to bring productivity to levels comparable to those of well-established platforms.

2.1.4 The “Hairy Root” Culture System

Together with cell suspensions, organ cultures such as hairy root offer advantages including containment, defined cultivation conditions and product homogeneity (Schillberg et al. 2013). Hairy roots (HR) are particularly attractive for the

industrial-scale production of secondary metabolites but also of pharmaceutical proteins. In fact, like undifferentiated cells, they grow in simple, defined media but are genetically stable and easy to handle, with protocols available to establish transgenic lines highly scalable, producing significant biomass accumulation (Guillon et al. 2006).

HR cultures are generated by *Agrobacterium rhizogenes* infection. This infection leads to the generation of root clones characterized by an extensive secondary branching that can be cultivated under contained sterile conditions in hormone-free media (Franconi et al. 2010). Importantly, recombinant proteins can be secreted in the culture medium facilitating the downstream purification processes (Guillon et al. 2006).

HR have been used to express a range of recombinant proteins, such as enzymes (Woods et al. 2008), vaccines (Skarjinskaia et al. 2013), monoclonal antibodies (Wongsamuth and Doran 1997) and anti-HIV microbicides (Drake et al. 2013).

2.1.5 The CRISPR-Cas9 System

Editing the genome of a plant without introducing foreign DNA into cells is now a reality. Programmable nucleases, like zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and RNA-guided endonucleases, have been used for genome editing in plant cells, amplifying the horizon of plant molecular farming (Li et al. 2013; Shan et al. 2013; Nekrasov et al. 2013). Recently, the CRISPR (clustered regularly interspaced short palindromic repeat)/Cas9 (CRISPR-associated) system has been successfully used in a wide range of plant species (Bortesi and Fischer 2015) but not in plant suspension cells. The system is based on a short RNA guide (sgRNA) which associates to the Cas9 endonuclease to create a double-stranded break (DSB) in the target genomic DNA. As a consequence, mutations are generated through either error-prone non-homologous end-joining (NHEJ) or homology-directed repair (HDR) of the intended cleavage site. NHEJ has been used to generate mutagenic insertions/deletions often leading to gene inactivation. The resulting changes at chromosomal target sites can be indistinguishable from naturally occurring genetic variation. However, the system can be used to introduce larger modifications and insertion of exogenous sequences. Moreover, similar to "traditional" gene insertion, if *A. tumefaciens* is used as a delivery vector of CRISPR-Cas9, the resulting genome-edited plants contain foreign DNA sequences, in addition to those that encode the programmable nucleases, in the host genome. On the other hand, the use of episomal plasmids to deliver these nucleases into plant cells has to take into account that transfected plasmids are rapidly degraded in cells by endogenous nucleases and that the resulting DNA fragments can be inserted at off-target sites in host cells (Kim et al. 2014).

The delivery of preassembled Cas9-gRNA ribonucleoproteins (RNPs) into plant cells has been shown to remove the possibility to introduce recombinant DNA into the host plant genome (Cho et al. 2013). Furthermore, it as has been shown that these premixed RNPs cleave target DNA sites immediately after transfection

and are degraded rapidly by endogenous proteases, thus reducing the frequency of off-target effects in regenerated plants (Kim et al. 2014; Cho et al. 2013). Moreover, when using protein-and-RNA-only systems, there is no need to optimize codon usage or to find promoters that will express Cas9 and gRNAs. In 2015, the delivery of RNPs into protoplasts of *Arabidopsis thaliana*, *Nicotiana attenuata* and *Oryza sativa* and the induction of targeted genome modifications in regenerated plants were reported. Purified Cas9 protein was mixed in molar excess with gRNAs targeting genes from the three plant species in vitro to obtain preassembled RNPs. The RNPs were incubated with protoplasts in the presence of polyethylene glycol. Indels were detected at the expected positions, with various frequencies (Woo et al. 2015).

2.2 *Transient Expression (Delivery of Genes to Somatic Tissues)*

The alternative plant-based technology to stable transformation of genomes is transient expression. As mentioned, stable transformation is not suitable for the rapid production of pharmaceuticals. This feature would be an obstacle to the use of plant-based technologies particularly in specific fields like those related to the production of biologics to combat emerging diseases.

The transient technological platforms imply the introduction of episomal vectors into plant tissues. There are two main transient expression strategies. The first is agro-infiltration, where leaves are infiltrated with *A. tumefaciens* (by injection or by applying vacuum to transfect whole plants at a glance). The second implies the infection of plant tissues directly with recombinant plant viruses that infect cells, replicate and spread from cell to cell and systemically guide the expression of recombinant protein in every cell. The majority of plant viral vectors used are based on single-stranded RNA viruses, such as tobacco mosaic virus, potato virus X and cowpea mosaic virus (CPMV) (Loh et al. 2017).

The use of deconstructed virus genomes delivered by *A. tumefaciens* is a methodology that is in between agro-infiltration and viral infection. The target gene is cloned into a modified plant viral vector which can be integrated into the *Agrobacterium* vector and delivered into the plant tissues by infiltrating with the corresponding transformed *Agrobacterium*. This facilitates the transfer of T-DNA to a very high number of cells. With the use of a deconstructed virus, the issue of instability of viral genome due to the introduction of large target genes can be resolved. Moreover, a certain degree of containment can be obtained because it implies the transfection of many cells with the virus genome followed by its cell-to-cell movement but no systemic spreading (Peyret and Lomonossoff 2015).

Icon Genetics, a German plant biotechnology company, has adapted this technology as MagnICON™ for the manufacturing of various plant-based vaccines, including those based on the hepatitis B virus surface antigen (300 mg/kg *N. benthamiana* fresh leaves) in the form of VLP (Huang et al. 2006), norovirus

capsid proteins (Scotti and Rybicki 2013; Rybicki 2014) and non-Hodgkin lymphoma vaccines, which proceeded to a phase I clinical trial (see also Tables 4 and 5) (McCormick et al. 2008).

Fraunhofer USA Center for Molecular Biotechnology (CMB) has developed a "launch vector", an advanced, hybrid, gene infiltration system that combines the elements of TMV vector and *A. tumefaciens* binary plasmids (Musiychuk et al. 2007). Among others developed, the launch vector pBID4 contains the 35S promoter from cauliflower mosaic virus (35S CaMV) that drives transcription of the viral genome, the nopaline synthase terminator, genes for virus replication and cell-to-cell movement and the target gene cloned under the transcriptional control of the coat protein subgenomic mRNA promoter. Following infiltration, primary transcripts produced in the nucleus are transported into the cytoplasm, resulting in robust protein production (such as 1 mg/g of fresh weight) (Musiychuk et al. 2007; Massa et al. 2007).

The pEAQ system is based on full-length or truncated versions of CPMV RNA-2 for efficient and rapid protein production without viral replication (Sainsbury et al. 2009). These vectors contain the 35S CaMV promoter, nos terminator, the p19 sequence encoding a suppressor of silencing and 5′- and 3′-UTRs from CPMV RNA-2. The gene of interest is inserted between the UTRs. The "HT" (hypertranslatable) variants of these family of vectors provide extremely high translational efficiency and, eventually, high level of the recombinant protein accumulation in plant biomass (Peyret and Lomonossoff 2015).

Transient expression of target proteins in plants is, therefore, considered a more feasible approach when compared to stable transformation, due to its rapid production capabilities often leading to the expression of large amounts of recombinant protein in a short time. The process is scalable just by virus-infecting or agro-infiltrating more plants.

Agro-infiltration has demonstrated the highest efficiency and highest levels of target protein expression with the potential for cost-effective production (Loh et al. 2017). *Nicotiana benthamiana* is particularly amenable to infiltration methods. The maximum of protein expression is generally observed within 7 days post-infiltration which is faster if compared to the mere virus strategy that requires at least 2 weeks to generate a systemic spreading and expression. As mentioned, yields using this approach can reach 1 g of product per kilogram of leaves, though the levels are obviously protein-dependent. Successful clinical trial has indicated safety and efficacy of the protein therapeutics and biologics made by agro-infiltrated plants (see Table 5). The main example is represented by the production in *Nicotiana benthamiana* of a vaccine candidate against H1N1 influenza pandemic that occurred in 2009: the Canadian company Medicago was able to produce a ready to be administered hemagglutinin-based vaccine by transient expression in 19 days (D'Aoust et al. 2010).

3 Plant-Made Antigens for Developing Vaccines

The production of plant-based pharmaceuticals is strongly motivated especially in the field of vaccines, which are the most effective tools for the prevention and treatment of infections and, in some cases, cancer. Unfortunately, the progress toward commercialization of vaccines takes the biggest effort and time compared to other biopharmaceuticals, and this is also the case of plant-based vaccine candidates.

The possibility of manufacturing recombinant biopharmaceutical proteins in plants initially gave the prospect of a possible vaccine delivery by edible plant material ("edible vaccines") (Haq et al. 1995). Nevertheless, the idea of vaccination through ingestion of raw plant material (e.g. fruits or tubers) turned out to be not feasible due to different issues (uppermost, uniformity in dosage). The concept of oral antigen delivery in processed plant material is now considered more feasible (Streatfield 2006). A limited number of clinical trials involving oral delivery of antigens have been performed. In all cases no major safety concerns were detected, and formulations turned out to be well tolerated by vaccinees.

The first trials were conducted with the non-toxic B subunit of heat-labile enterotoxin (LTB) of enterotoxigenic *E. coli* contained either in raw potato or maize administered orally to healthy volunteers for safety and immunogenicity testing. No adverse effects of vaccination were noticed. LTB-specific IgA-secreting cells in peripheral blood and increased levels of LTB-specific serum IgG and IgA were detected after vaccination (Tacket et al. 1998; Tacket 2007).

Norovirus capsid protein VP1 was produced in potato tubers. Vaccination with approximately 500 μg of recombinant VP1 was done. Despite the high infectivity of the virus resulting in pre-existing serum immunity in a portion of volunteers, vaccinees developed VP1-specific serum IgG titres (Tacket et al. 2000).

Also a pathogen like the hepatitis B virus (HBV), able to evolve chronic infection and cancer both in adults and in infants, was targeted by PMF. HBV surface antigen (HBsAg) transgenic lettuce leaves containing 0.1–0.5 μg of HBsAg/100 g of fresh tissue were given to three adult volunteers showing transient protective levels of specific IgG after vaccination, but no HBsAg-specific serum IgA (Kapusta et al. 1999). When HBsAg was produced in transgenic potato (Thanavala et al. 2005), a dose-dependent, elevated serum HBsAg antibody titre induction was observed after administration of 850 ± 210 μg of antigen/dose, in a clinical trial on volunteers over the 70-day follow-up period after the first immunization.

Fragments encoding a chimeric protein of G protein and N protein of the rabies virus fused with that of alfalfa mosaic virus coat protein were introduced into a TMV-derived plant expression vector. The vaccine transiently expressed in spinach was orally administered (three doses of spinach; 20 g corresponding to 84 μg of chimeric rabies peptide each) in a clinical trial revealing the induction of elevated rabies-specific IgG in a quote of both in individuals previously vaccinated with a commercial injection-type vaccine and in volunteers with no history of rabies vaccination (Yusibov et al. 2002).

Overall, these studies have indicated that an immune response can be mounted in individuals fed plant material expressing a disease antigen.

Several other antigens have been expressed through different methodologies to the purpose of manufacturing (nonedible) candidate vaccines intended for clinical (Tables 3 and 4) and veterinary use (Table 5). Plant-made antigens targeting various pathogens have been shown to be effective in animal models (Table 3). Many of these candidates have reached phases I–II human clinical trials (Table 4). Safety of these vaccines was demonstrated; nevertheless, at present, there are no plant-made vaccine antigens approved for clinical use.

Despite this, plant-based expression platforms demonstrated to be able to tackle important sanitary problems even in emergency contexts. As already explained, in emergency, the transient methods assure the shortest leading times to the final products. That's why the major examples of plant-made vaccines in clinical trial are from transient approaches (Table 4). As an example, influenza virus, a very complex pathogen with 16 different hemagglutinin (HA) subtypes and characterized by occurrence of antigenic shift abolishing cross-protective immunity of the host even against strains of the same subtype, has the potential to be pandemic as happened in 2009 for the H1N1-type influenza virus. To control its diffusion by vaccination, fast production of a new HA antigen was needed. Medicago Inc. was able to develop a technology obtaining the accumulation of HA virus-like particles (virus-like particles, VLPs) in the apoplast of *N. benthamiana* cells by transient expression (D'Aoust et al. 2010; US patent application number 20130183341). Phase I (5, 10 or 20 μg of H5-VLP subcutaneously injected twice with alum adjuvant) and II (20, 30 or 45 μg of H5-VLP) clinical trial of the VLP composed of HA protein of H5N1 influenza virus (A/Indonesia/5/05) has showed, respectively, the induction of hemagglutinin inhibition titres at all tested doses and cross-protective CD4+ T-cell responses, indicating strong induction of long-term cell-mediated immunity by plant-made H5-VLP after 6 months of vaccination (Landry et al. 2012). No detectable IgE responses (and, therefore, allergy or hypersensitivity) against plant-specific mannose residues were found, one of the main issues that are often risen against the production of protein-based pharmaceuticals in plant-based systems (Ward et al. 2014). Medicago Inc. performed also a phase I clinical trial with 5, 13 or 28 μg of H1N1 influenza (A/California/7/09) VLPs that demonstrated safety and induced immune response to the virus, including cell-mediated immunity (Landry et al. 2010).

Other plant-based influenza vaccines (HA from A/California/04/2009 H1N1 (HAC1) and A/Indonesia/05/05 H5N1 (HAI-05); Fraunhofer CMB USA) based on transient expression have successfully completed phase I clinical trial (Shoji et al. 2011, 2015).

Plant-based platforms have been identified also as promising approaches to tackle chikungunya virus, the emerging pathogen initially found in East Africa and currently spread in many regions of the world. No licensed vaccines are available, and thus the need for advancing in this research field is great. Beside the preclinical stage candidates in trial, also the implementation in parallel of low-cost production platforms will be determinant as chikungunya is mainly affecting developing countries where access to vaccines is limited due to the high cost of conventional formulations. Based on the previous experiences on influenza and other viral pathogens, plant-made VLPs seem the most efficacious approach to render immunogenic formulations also in this case (Salazar-González et al. 2015).

Table 3 Examples of plant-derived vaccines for the prevention and treatment of human diseases (sourced from Loh et al. 2017)

Target protein	Host/expression strategy	Functionality evaluation	Reference
Anthrax protective antigen 83 (PA83)	*Nicotiana benthamiana*/ transient	Detection of high-titre toxin-neutralizing antibodies. 100% survival of immunized rabbits (IM) against lethal anthrax challenge	Chichester et al. (2013)
	Brassica juncea (mustard)/transgenic (nuclear)	Detection of systemic and mucosal immune responses. 60% survival of orally immunized mice against lethal anthrax challenge	Gorantala et al. (2014)
	Nicotiana tabacum (tobacco)/transgenic (chloroplast)	Detection of systemic and mucosal immune responses. 80% survival of orally immunized mice against lethal anthrax challenge	Gorantala et al. (2014)
Dengue consensus domain III of envelope glycoprotein (cEDIII) with 6D8 anti-Ebola IgG	*N. benthamiana*/ transient	Detection of virus-neutralizing specific anti-cEDIII humoral immune response in immunized mice (SC)	Kim et al. (2015)
Ebola glycoprotein (GP) in fusion with 6D8 anti-Ebola IgG	*N. benthamiana*/ transient	Detection of humoral immune responses. 80% survival of immunized mice (SC) against lethal EBV challenge	Phoolcharoen et al. (2011)
EBOV GP1 in fusion with E. coli heat-labile enterotoxin B subunit	Tobacco/transgenic (nuclear)	Detection of serum IgG in immunized mice (SC) and faecal IgA in immunized mice via oral administration	Rios-Huerta et al. (2017)
Hepatitis B virus (HBV) small surface antigen	*Lactuca sativa* (lettuce)/transgenic (nuclear)	Detection of serum IgG in immunized mice via oral administration	Czyż et al. (2014)
HBV surface antigen (HBsAg) SUV against HBV	*Solanum tuberosum* (potato)/transgenic (nuclear)	Induction of serum antibodies and stable immunological memory in immunized mice fed with transgenic potato tubers	Rukavtsova et al. (2015)
Human immunodeficiency virus (HIV) gp120 multi-epitopic envelope protein	Lettuce/transgenic (nuclear)	Detection of cell-mediated and humoral immunities in immunized mice via oral administration	Govea-Alonso et al. (2013)
HIV gp120 and gp41 multi-epitopic envelope proteins	Tobacco/transgenic (chloroplast)	Detection of antibody and cellular responses as well as specific IFN-g production in immunized mice via oral administration	Rubio-Infante et al. (2015)

(continued)

Table 3 (continued)

Target protein	Host/expression strategy	Functionality evaluation	Reference
HIV-1 envelope proteins (Gag/Dgp41) VLPs	*N. benthamiana*/ transient	Induced Gag-specific serum antibody and CD4 and CD8 T-cell responses in mice via systemic (IP) and mucosal (IN) immunizations	Kessans et al. (2016)
Human papillomavirus type 16 (HPV-16) HPV-16L1	Tobacco/ transgenic (nuclear)	Detection of cell-mediated and humoral immunities in immunized mice via oral administration	Hongli et al. (2013)
HPV-16 E6 and E7 fusion (HPV-16L1 E6/E7) VLP	*Solanum lycopersicum* (tomato)/transgenic (nuclear)	Detection of persistent neutralizing antibodies and 57% tumour reduction in immunized mice via oral administration	Monroy-García et al. (2014)
HPV-16 E7	*N. benthamiana* (transient)	Detection of antibodies and cell-mediated responses, tumour reduction in immunized mice models via subcutaneous administration	Massa et al. (2007); Venuti et al. (2009)
Influenza H1N1 HA from A/California/04/09 strain (HAC-VLPs)	*N. benthamiana*/ transient	Detection of serum HI antibody responses in immunized mice (IM)	Shoji et al. (2013)
Influenza H3N2 nucleoprotein	*Zea mays* (maize)/ transgenic (nuclear)	Detection of humoral immune responses in immunized mice via oral administration	Nahampun et al. (2015)
Influenza H5N1 HA1 domain (HA1-MY)	*N. benthamiana*/ transient	Detection of serum HI antibody responses in immunized mice (IM)	Pua et al. (2017)
Rabies virus glycoprotein in fusion with ricin toxin B chain	*Solanum lycopersicum* (tomato) hairy roots/transgenic (nuclear)	Detection of serum IgG and Th2 lymphocyte responses in immunized mice via intra-mucosal administration	Singh et al. (2015)
Severe acute respiratory syndrome coronavirus (SARS-CoV) nucleocapsid (N) protein	*N. benthamiana*/ transient	Recognition of SARS patient sera by purified N protein	Demurtas et al. (2016)

After pioneering examples that we have already mentioned in the field of "edible vaccines" (e.g. against hepatitis B), and others undertaken against human immunodeficiency virus (in lettuce, tobacco) and human papillomavirus (in tomato) as main cases, stable transformation approaches (both nuclear and plastidial) are also currently being undertaken and evaluated in preclinical context to tackle highly relevant sanitary problems such as those related to deadly pathogens like anthrax (in mustard and tobacco) and Ebola virus (tobacco) (see Table 3).

Table 4 Recent examples of plant-based vaccine antigens at various stages of clinical trials (sourced from Loh et al. 2017)

Product/Application	Plant host	Status	Company (sponsor)
Pfs25 VLP-FhCMB Malaria transmission blocking vaccine	*N. benthamiana*	NCT02013687 Phase I (completed in 2015)	FhCMB, USA
PA83-FhCMB Against anthrax	*N. benthamiana*	NCT02239172 Phase I (completed in 2015)	FhCMB, USA
HAC1 H1N1 seasonal influenza virus	*N. benthamiana*	NCT01177202 Phase I (completed in 2012)	FhCMB, USA
HAI-05 H5N1 pandemic influenza virus	*N. benthamiana*	NCT01250795 Phase I (completed in 2011)	FhCMB, USA
H1 (VLP) H1N1 seasonal influenza virus	*N. benthamiana*	NCT01302990 Phase I (completed in 2011)	Medicago, Canada
Quadrivalent VLP H1N1, H3N2 seasonal influenza B viruses	*N. benthamiana*	NCT01991587 Phases I and II (completed in 2014)	Medicago, Canada
Quadrivalent VLP H1N1, H3N2, seasonal influenza B viruses	*N. benthamiana*	NCT02233816 Phase II	Medicago, Canada
Quadrivalent VLP H1N1, H3N2, seasonal influenza B viruses	*N. benthamiana*	NCT02236052 Phase II	Medicago, Canada
H5 VLP H5N1 pandemic influenza virus	*N. benthamiana*	NCT00984945 Phase I (completed in 2010)	Medicago, Canada
H5 VLP H5N1 pandemic influenza virus	*N. benthamiana*	NCT01244867 Phase II (completed in 2011)	Medicago, Canada
H5 VLP H5N1 pandemic influenza virus	*N. benthamiana*	NCT01991561 Phase II (completed in 2014)	Medicago, Canada
H5-VLP + GLA-AF H5N1 pandemic influenza virus	*N. benthamiana*	NCT01657929 Phase I (completed in 2014)	Medicago (IDRI), Canada
H7 VLP H7N9 pandemic influenza virus	*N. benthamiana*	NCT02022163 Phase I (completed in 2014)	Medicago, Canada

Unfortunately, at present, the only plant-made vaccine approved is a purified and injectable Newcastle disease vaccine for poultry produced in tobacco suspension cells (Rybicki 2010, Table 6). The approval of this veterinary plant-made vaccine represents a quite important milestone. It should be considered that plant-based technologies are very suitable for developing veterinary vaccines. Especially in the

Table 5 Plant-based vaccine antigens for veterinary use

Product/application	Plant host	Reference/status
Chicken Newcastle disease Haemagglutinin-neuraminidase	Tobacco suspension cells	Katsnelson et al. (2006) Approved by USDA
Chicken Newcastle disease F protein	Maize	Guerrero-Andrade and Loza-Rubio (2006)
Chicken infectious bronchitis virus (IBV) S1 glycoprotein	Potato	Zhou et al. (2004)
Chicken IBV VP2	Rice	Wu et al. (2007)
Pig enterotoxigenic *E. coli* (ETEC) Fimbriae (F4)	Tobacco (chloroplast)	Kolotilin et al. (2012)
Pig ETEC Fimbriae (F4)	Alfalfa	Joensuu et al. (2006)
Pig ETEC Cholera toxin B subunit	Rice	Takeyama et al. (2015a, b)
Pig foot-and-mouth disease virus VP1	*N. benthamiana*	Yang et al. (2007)
Pig porcine transmissible gastroenteritis virus Spike protein	Tobacco	Tuboly et al. (2000)
Cattle bovine herpesvirus gD protein	Tobacco	Pérez Filgueira et al. (2003)
Cattle bovine viral diarrhoea virus E2 protein	Alfalfa	Peréz Aguirreburualde et al. (2013)
Cattle rinderpest virus Hemagglutinin	Peanut	Khandelwal et al. (2003)

case of fisheries, since farming relies on ocean water and excessive use of antibiotics may have impact on the environment, the use of (edible) vaccines for disease prevention may mitigate this problem (Clarke et al. 2013).

4 Plantibodies

Antibodies represent a peculiar example of biopharmaceuticals that can be efficiently expressed utilizing plants as low-cost heterologous hosts. In fact, prokaryotic expression systems are generally inappropriate for complete antibodies, due to the lack of some post-translational modifications such as *N*-linked glycosylation, which is often required for therapeutic proteins to be effective, and the inconsistency of others like the stabilization of free sulphydryls (i.e. from cysteines) and the reduction of disulphide bonds necessary for peptides and proteins to fold correctly.

As already mentioned, in the past decades, after the first, impressive evidence of correct expression and assembly of a full immunoglobulin in plant cells (Hiatt et al. 1989), unimaginable before the early 1990s, plant-made antibodies have been

Table 6 Recent examples of plant-based antibodies against tumour malignancies and infectious diseases

Antibody	Host/expression strategy	Functionality evaluation	Reference
Trastuzumab (humanized)	*N. benthamiana* (transient)	Anti-proliferative effects, similarly to the current drug Herceptin	Komarova et al. (2011); Grohs et al. (2010)
Rituximab (BLX-300 mouse/human chimeric anti-CD20 antibody C2B, with human-like glycosylation)	Aquatic plant *Lemna minor* (LEX System)	In preclinical studies showed the same target cell-binding activity, improved antibody-dependent cellular cytotoxicity against B cells and superior B-cell depletion, induction of apoptosis but attenuated complement-dependent cytotoxicity	Cox et al. (2006); Gasdaska et al. (2012)
Rituximab immunocytokine (scFv-Fc fused to human IL-2 with homogeneous glycosylation devoid of xylose/fucose *N*-glycosylation)	*N. benthamiana* (transient)	In vitro CD20 binding activity comparable to Rituximab and efficient elicitation of antibody-dependent cell-mediated cytotoxicity (ADCC) hIL-2 in the context of 2B8-Fc-hIL-2 maintained the biological activity and subsequent triggering of T-cell proliferation	Marusic et al. (2016)
Obinutuzumab (humanized from mouse monoclonal antibody against a CD20 epitope partially overlapping that of rituximab and glycoengineered)	*N. benthamiana* (transient)	Substantially equivalent to that derived from conventional Chinese hamster ovary (CHO) cells in binding to CD20 and expanding the immune-mediated target cell death	Lee et al. (2018)
2G12 (human mAb against a neutralizing epitope on HIV-1 GP120 protein)	Tobacco, maize and *Arabidopsis* (transgenic)	Potent and broad HIV-1-neutralizing activity in vitro and in vivo	Hessell et al. (2009)
		Double-blind, placebo-controlled, randomized, dose-escalation, phase I clinical trial was performed	Ma et al. (2015)
	Tobacco (transient)	Binding and neutralization properties with the "humanized" *N*-glycans equivalent to that of the original mAb derived from CHO cells. More effective in virus neutralization	Sainsbury et al. (2010)
	N. tabacum and *N. benthamiana* (transgenic, sIgA version of 2G12)	Effective aggregation of HIV virions and enhanced stability in mucosal secretions when compared to the cognate IgG format	Paul et al. (2014)

(continued)

Table 6 (continued)

Antibody	Host/expression strategy	Functionality evaluation	Reference
Paramyxovirus respiratory syncytial virus humanized IgG1 mAb palivizumab (different isotypes and *N*-glycoform variants)	*N. benthamiana* (transient) also utilizing glico-engineered plant hosts	Compared to commercially available palivizumab with respect to both in vitro receptor and C1q binding and in vivo efficacy. Antigen binding and neutralization activity of each variant were indistinguishable from those of palivizumab. Fcγ receptor binding profiles demonstrated significant differences depending on isotype and glycan profile	Hiatt et al. (2014)
Human mAb against anthrax main virulence factor(PA)("humanized", glycosylated with plant-specific glycans or glycan-deprived)	*N. benthamiana* (transient)	Used in both prophylactic and therapeutic treatment of individuals exposed or infected with anthrax. Both formats were able to bind PA, neutralizing anthrax lethal toxin, and to protect mice against a lethal spore challenge. The glycan-deprived mAb demonstrated improved half-life providing full protection in non-human primates against anthrax spore inhalation	Mett et al. (2011)
Chimeric complete IgG and a IgG-like format (scFv-Fc) against opportunistic fungal pathogens derived from a murine mAb (2G8)	*N. benthamiana* (transient)	Retained the efficacy of the original mAb, being able to inhibit directly the growth of *Candida albicans* and to induce the protection in animal models mimicking a systemic or vaginal *C. albicans* infection	Capodicasa et al. (2011)
		Both Abs promoted the killing of fungal cells by human polymorphonuclear neutrophils in ex vivo assays In a further development, a recombinant IgA version was also expressed and correctly assembled in dimeric form in plants	Capodicasa et al. (2017)
IgG against Ebola virus GP1	*N. benthamiana* (transient)	Protective against infection	Huang et al. (2010)
ZMapp (MB-003 mAb cocktail consisting of three human and human-mouse chimeric mAbs c13C6, h13F6 and c6D8 produced plants)	*N. benthamiana* (transient)	Three times as effective as CHO cell-produced recombinant counterpart mAbs, most likely due to the absence of core fucose residues on the Fc region of the plant-derived mAbs, which results in enhanced antibody-dependent cellular cytotoxicity	Zeitlin et al. (2011)

(continued)

Table 6 (continued)

Antibody	Host/expression strategy	Functionality evaluation	Reference
ZMapp cocktail, consisting of selected components from MB-003 (c13C6) and ZMAb (humanized c2G4 and c4G7)	*N. benthamiana* line XT/FT (transient)	Compassionate use of experimental interventions to seven Ebola patients as a post-exposure therapy. Of these patients, five significantly improved and recovered from the disease even though the treatment was initiated at least 9 days after infection A randomized phase I/II clinical trial of ZMapp as a putative investigational therapeutic in the treatment of patients with known Ebola infection started in 2015	Qiu et al. (2014)

promoted and validated as one of the most promising plant cell-based biologics. In fact, preclinical and current clinical trials hold promise for immunotherapy based on these recombinant molecules with peerless safety and economic qualities.

The recent success gained against Ebola virus by ZMapp™, a cocktail of three mouse/human chimeric antibodies, developed and produced in *N. benthamiana* by Mapp Biopharmaceutical Inc. (Davey Jr et al. 2016), shed new light on the potential of these "green" recombinant molecules as novel products of the healthcare system, possibly in the context of the "biosimilars" or "biobetters" class, able to lower the barriers to patients' access to treatment. To this end, it should be considered that some of the original antibody-based drugs (i.e. those from the 1980s and 1990s) are or will soon be coming off patent, and, therefore, they may attract investments in the development of novel industrial facilities to meet the increasing demand of these drugs. Indeed, at the moment, all protein-based active pharmaceutical ingredients derived from plants are still outlandish drugs for most pharmaceutical industries, although these herbal reagents would be competitive thanks to flexible and very large-scale production capacity, especially when the profitability ratios of the mammalian cell counterpart falter (Whaley et al. 2014).

4.1 Plant-Made Antibodies in Cancer Diagnostics and Therapy

Monoclonal antibodies (mAbs) are highly effective pharmaceuticals in cancer therapy (Gaughan 2016) with an estimated market value of $150 billion by 2021 (https://www.researchandmarkets.com/research/b3gj4m/the_development) (Ecker et al. 2015). The demand for some major products is in the order of tonne-scale production in the developed countries. mAbs' consumption may even increase if the supply of these biopharmaceuticals is to be extended to developing countries,

which currently have limited access to these biologics, due to the current costs of manufacturing in mammalian cell culture. This scenario would unlock the full potential of plants as a novel production platform.

mAbs directed to several tumour-associated antigens (Table 6) and involved in different mechanisms of interference/diagnosis of the relevant cancer type have been expressed in both plant leaves and seeds (Pujol et al. 2007). The genes for heavy and light chain of the base format of the immunoglobulin have been engineered for transient or stable expression through different techniques of transgenesis involving both nuclear and chloroplast genome (Ko et al. 2005; Komarova et al. 2010; Daniell 2003).

As mentioned elsewhere in this chapter, every expression technique (either stable or transient) presents advantages and drawbacks. Therefore, a careful evaluation of the system has to be adopted on a case-by-case basis. Nevertheless, while cumbersome procedures are required for the establishment of stable genome transgenic lines and literature highlights expression yields usually non-competitive (De Muynck et al. 2010), transient expression systems allow high yields of recombinant protein in few days. The transient approach has been prevalent in recent years in an attempt to make the plant system highly productive for the synthesis of biologic medicines. As an example, by this approach it is currently possible to produce ten million doses of influenza vaccine in the remarkable time of 6 weeks (Pillet et al. 2016).

In a pioneering work of agro-infiltration, a clinically relevant recombinant antibody (diabody) against the carcinoembryonic antigen (CEA) in tumour imaging was expressed in tobacco plants (Vaquero et al. 2002) validating the feasibility of the approach. In subsequent study, 500 mg/kg yields of a full-size tumour-specific human mAb A5 were achieved using the "MagnICON" system (magnifection) developed by Icon Genetics (Giritch et al. 2006; Marillonnet et al. 2004).

Since the discovery of competitive manufacturing yields, anticancer therapeutic antibodies produced as plant biosimilars occupy an area of active interest due to the potential to guarantee access to more affordable treatments also in view of patents' expiry dates for quite a number of antibodies by 2020. The following examples witness a fervent activity in this sense.

TheraCIM, a recombinant humanized antibody against the epidermal growth factor receptor (EGF-R) that had orphan drug status for glioma, starting from 2014 in the United States and EU, was one of the first antibodies of commercial value to be transiently expressed in plants. Engineered in an aglycosylated format in tobacco, this antibody, produced from agro-infiltrated leaves, maintained unchanged the recognition of the EGF-R on the surface of human tumour cultured cells (Rodriguez et al. 2005).

Trastuzumab, a humanized monoclonal approved by the US Food and Drug Administration for the treatment of metastatic breast cancer, recognizing the human epidermal growth factor receptor 2 (HER2/neu), has been transiently expressed in *N. benthamiana* plants (Komarova et al. 2011; Grohs et al. 2010). In these independent reports, this plant-made mAb, expressed using either the

MagnICON system or tobacco mosaic virus- and potato virus X-based vectors, revealed anti-proliferative effects, similarly to the current drug Herceptin.

The mouse/human chimeric anti-CD20 antibody C2B8 (rituximab) is the first antibody-based drug approved by regulatory authorities for the treatment of patients with recurrent B-cell lymphomas and is capable of depleting B cells in vivo (Reff et al. 1994). Although only about 48% of patients treated with rituximab respond to the therapy, with <10% showing a complete remission of the tumour (Davis et al. 2014), treatment with this drug associated with chemotherapy is a pillar for the treatment of non-Hodgkin's lymphomas (Plosker and Figgitt 2003).

In an effort to increase cost-effectiveness of rituximab, Biolex Therapeutics used the proprietary protein expression (LEX System) in the aquatic plant *Lemna minor* to produce a plant version of rituximab with fully human-like glycosylation pattern with a single major *N*-linked glycan devoid of xylose or fucose (Cox et al. 2006). This new product, named BLX-300, was shown to have the same target cell-binding activity, improved antibody-dependent cellular cytotoxicity against B cells and superior B-cell depletion, induction of apoptosis but attenuated complement-dependent cytotoxicity in preclinical studies (Gasdaska et al. 2012).

A further approach to improve efficacy of therapy of B-cell malignancies is based on the use of anti-CD20 antibodies to deliver cytokines to the tumour microenvironment. In particular, human interleukin 2 (IL-2)-based immunocytokines have shown enhanced antitumour activity in several preclinical studies (List and Neri 2013).

The first successful example of a recombinant immunocytokine produced in agro-infiltrated *N. benthamiana* plants was reported in an unconventional yet effective approach (Marusic et al. 2016). The immunocytokine was designed as a protein fusion between an antibody scaffold derived from rituximab and the human IL-2. In particular, a dimeric bivalent antibody format based on a scFv-Fc fused to IL-2 with highly homogeneous glycosylation devoid of the typical xylose/fucose *N*-glycosylation plant signature. In vitro studies showed that this antibody conjugate has a CD20 binding activity comparable to that of rituximab and an efficient elicitation of antibody-dependent cell-mediated cytotoxicity (ADCC). In addition, hIL-2 in the context of 2B8-Fc-hIL-2 maintained the biological activity, as validated by 2B8-Fc-hIL-2 binding to the hIL-2 receptor and subsequent triggering of T-cell proliferation. The final touch was the discovery that glycan engineering, performed utilizing RNAi-silenced transgenic plants devoid of xylosyltransferase and fucosyltransferase activity (Strasser et al. 2008), was the best way to achieve an overall improvement of the biological functions (Marusic et al. 2018).

Among antibodies targeting B-cell malignancies, obinutuzumab is a third generation fully humanized from mouse monoclonal antibody that binds a CD20 epitope partially overlapping that of rituximab (Klein et al. 2013). Obtained through a proprietary GlycoMAb® technology by GlycArt Biotechnology and developed by Roche as a therapeutic of chronic lymphocytic leukaemia and other B-cell non-Hodgkin's lymphomas (Marcus et al. 2017), this glycoengineered mAb, when produced in *N. benthamiana* plants, has been demonstrated substantially equivalent to that derived from conventional Chinese hamster ovary (CHO) cells in binding to CD20 and expanding the immune-mediated target cell death (Lee et al. 2018).

4.2 Plant-Made Antibodies and Immunotherapy of Infectious Diseases

Numerous mAbs have shown the potential to treat infectious diseases targeting microbial infections by directly interacting with key epitopes and/or harnessing the immune response.

Among anti-infectious mAbs, the human mAb 2G12, recognizing a distinctive neutralizing epitope on the GP120 envelope protein of HIV-1, has a potent and broad HIV-1-neutralizing activity in vitro and in vivo (Hessell et al. 2009). Due to these properties, mAb 2G12 has been chosen by Pharma-Planta Consortium (http://www.pharma-planta.net) as the best candidate to be produced in plants under GMP conditions for the first in-human, double-blind, placebo-controlled, randomized, dose-escalation, phase I clinical trial (Ma et al. 2015). This mAb, representing a significant milestone in the commercial development of plant-derived biologics, has been produced by several expression systems and transgenic hosts (i.e. tobacco, maize and *Arabidopsis*) as well as in plant cell systems. However, the production of this mAb in transient expression systems was far superior to that obtained by stable transgenics in terms of yield and speed of expression. In particular, the highest yield of purified mAb (105 mg/kg fresh weight tissue) was obtained through the CPMV-HT system and retention in the ER (Sainsbury et al. 2010). In addition, binding and neutralization properties of plant-produced 2G12 with the "humanized" *N*-glycans were equivalent to that of the original mAb derived from CHO cells, being even more effective in virus neutralization.

Finally, a sIgA version of 2G12 has been produced in both transgenic *N. tabacum* and transiently transformed *N. benthamiana* (Paul et al. 2014) demonstrating effective aggregation of HIV virions and enhanced stability in mucosal secretions, when compared to the cognate IgG format.

A further antiviral mAb expressed in plants is the Hu-E16, a humanized mAb that binds an epitope on the envelope protein (domain III of GP-E) of West Nile virus (WNV) (Oliphant et al. 2005). Using both the MagnICON system and a geminivirus-based vector, codon-optimized HC and LC encoding sequences, high level of production (~260/800 and mg per kilogram of fresh weight) has been achieved in *N. benthamiana* and lettuce (Lai et al. 2010, 2012).

Further efforts have been made to improve Hu-E16 mAb efficacy, engineering diverse modified formats to be transiently expressed in both wild-type and glyco-engineered XT/FT *N. benthamiana* (He et al. 2014; Lai et al. 2014). Overall data demonstrate that plant-made Hu-E16 show in mice a strong neutralizing activity and induced pre- and post-exposure protection, equivalently to what has been observed with Hu-E16 derived from mammalian cell, possibly transforming this preclinical candidate into a cost-effective therapeutic of medical and veterinary significance against WNV.

Most current experimental therapies against Ebola virus (EBOV) are addressed to the principal virulence factor GP, a transmembrane protein triggering virus host cell entry and exerting cytopathic effects in infected cells (Lee et al. 2008; Simmons et al. 2002) while inducing protective antibodies (Wilson et al. 2000). At the moment,

the most advanced Ebola treatment is based on antibody-mediated therapy. In an early attempt, remarkable expression levels (0.5 mg per gram of fresh tissue) of a protective IgG against Ebola virus GP1, harnessing the geminivirus DNA replicon of bean yellow dwarf virus, were reported (Huang et al. 2010). At that time, the multi-replicon vector represented a significant advance in transient expression technology for antibody production in plants.

As previously illustrated, Mapp Biopharmaceutical, Inc., is involved in a research to find the best immunotherapy against Ebola, and the company has tested the drug MB-003, a mAb cocktail consisting of three human and human-mouse chimeric mAbs c13C6, h13F6 and c6D8 produced in CHO cells (Olinger Jr et al. 2012). When produced from plants, MB-003 resulted three times as effective as CHO cell-produced recombinant counterpart mAbs, most likely due to the absence of core fucose residues on the Fc region of the plant-derived mAbs, which results in enhanced antibody-dependent cellular cytotoxicity (Zeitlin et al. 2011). Nowadays the most advanced, optimized anti-Ebola mAb composition is the ZMapp cocktail, consisting of selected components from MB-003 (c13C6) and ZMAb (humanized c2G4 and c4G7) (Qiu et al. 2014). Using Magnifection, ZMapp was entirely produced in the glycoengineered *N. benthamiana* line ΔXT/FT (Strasser et al. 2008) as a product of LeafBio (San Diego, California, USA), a joint venture of Mapp Biopharmaceutical and Defyrus (Toronto, Canada) and was manufactured under cGMP at the Kentucky BioProcessing facility.

Although ZMapp safety had not been previously evaluated in humans, during the 2014–2015 Ebola outbreak in West Africa, it was administered outside clinical trials in compassionate use of experimental interventions to seven Ebola patients as a post-exposure therapy. Of these patients, five significantly improved and recovered from the disease even though the treatment was initiated at least 9 days after infection.

Nevertheless, a randomized phase I/II clinical trial of ZMapp as a putative investigational therapeutic in the treatment of patients with known Ebola infection is currently underway (ClinicalTrials.gov. 2015. Putative investigational therapeutics in the treatment of patients with known Ebola infection. Stud. Rec. NCT02363322, Natl. Inst. Health, Bethesda, MD. https://clinicaltrials.gov/ct2/show/NCT0236332237).

Today's state of the art indicates ZMapp as an effective drug for Ebola patients; however its supply is limited. Providing sufficient quantities of this biological is challenging due to the high dosage required for optimal therapy (Qiu et al. 2014) and to the limited capacity of the plant production system. Nevertheless, Mapp Biopharmaceutical has signed a contract with the US Department of Health and Human Services with the aim to accelerate the drug's development (McCarthy 2014).

Other plant biopharming companies are joining the global efforts against Ebola. Medicago, (Quebec, Canada), in the framework of a task order from the Biomedical Advanced Research and Development Authority at the US Department of Health and Human Services, is producing three anti-Ebola virus monoclonal antibodies (mAbs) with expected performance comparable to that of ZMapp™. In parallel, the Fraunhofer USA Center for Molecular Biotechnology is producing these anti-EBOV mAbs for the Biomedical Advanced Research and Development Authority, and

Caliber Biotherapeutics LLC declares itself ready to produce commercial quantities of ZMapp mAbs quickly and cost-effectively. Overall efforts will potentially boost production amounts and worldwide supply of these important drugs.

The paramyxovirus respiratory syncytial virus (RSV) can cause devastating lower respiratory tract infections in preterm infants and small children or elderly patients when other serious health problems are present. Passive immunization is an effective immunoprophylaxis against RSV, and treatment with a humanized IgG1 mAb, palivizumab (brand name Synagis, manufactured by MedImmune), is the current standard of care. In a recent report, plant-made different isotypes and *N*-glycoform variants were compared with commercially available palivizumab with respect to both in vitro receptor and C1q binding and in vivo efficacy. Whereas the antigen binding and neutralization activity of each variant were indistinguishable from those of palivizumab, their Fcγ receptor binding profiles demonstrated significant differences. Overall results indicate that interaction with FcγR, hence antibody-dependent cell-mediated cytotoxicity and not virus neutralization, plays the major role in palivizumab's efficacy. Therefore isotype and glycan profile engineering utilizing glycoengineered plant hosts have been put into play, paving the way for enhancing antibody-dependent cellular cytotoxicity and the efficacy of these anti-RSV mAbs (with yields of ~200 mg per kilogram of plant biomass) (Hiatt et al. 2014).

Anthrax is a zoonotic disease caused by the gram-positive, spore-forming bacterium *Bacillus anthracis.* Although the incidence of the disease has continually decreased since the late nineteenth century, anthrax is considered one of major concerns in the era of bioterrorism in that inhalation of aerosolized spores has very high mortality rates. *B. anthracis* possesses a main virulence factor, the 83-kDa form of protective antigen (PA), composed of two exotoxins, also known as lethal toxin and oedema toxin, which cause cell death following introduction into the cytoplasm (Young and Collier 2007). PA elicits specific neutralizing antibodies and is the major target for the development of both anthrax vaccines and mAbs. A human mAb against the *B. anthracis* PA has been overexpressed in plants to be used in both prophylactic and therapeutic treatments of individuals exposed or infected with anthrax. A humanized glycosylated (e.g. "decorated" with plant-specific glycans) and a glycan-deprived version of this mAb have been devised. Both formats were able to bind PA, neutralizing anthrax lethal toxin, and to protect mice against a lethal spore challenge. Surprisingly, the glycan-deprived mAb demonstrated improved half-life providing full protection in non-human primates against anthrax spore inhalation (Mett et al. 2011).

Infections caused by opportunistic fungal pathogens pose serious issues for both prevention and treatment especially in immune-compromised or hospitalized individuals. In the perspective of being used as a wide-spectrum antifungal therapeutics, a chimeric complete IgG and IgG-like format (scFv-Fc) have been produced in agro-infiltrated *N. benthamiana* plants (Capodicasa et al. 2011). These engineered Abs were derived from a murine mAb (2G8) that inhibits fungi growth conferring significant protection against different opportunistic pathogens such as *Candida albicans*, *Aspergillus fumigatus* and *Cryptococcus neoformans* in animal models (Torosantucci et al. 2009). By binding to a fungal cell wall polysaccharide

(β-glucan), both 2G8 chimeric IgG and scFv-Fc produced in plants retained the efficacy of the original mAb, being able to inhibit directly the growth of *Candida albicans* and to induce protection in animal models mimicking a systemic or vaginal *C. albicans* infection. In addition, both Abs promoted the killing of fungal cells by human polymorphonuclear neutrophils in ex vivo assays. In a further development, a recombinant IgA version was also expressed and correctly assembled in dimeric form in plants (Capodicasa et al. 2017). These recombinant Abs may be exploited to tackle a broad range of human fungal infections, in alternative or in addition to classical chemotherapy (Table 6).

5 Plant-Made Proteins for Other Pharmacological Uses

Currently, only two products derived from this technology have been brought on the market. The first, Elelyso, is an enzyme replacement therapy (ERT) for humans, while the second, Interberry-alpha, has been developed for veterinary use (canine gingivitis).

In 2012, the US FDA approved ELELYSO™ (human recombinant taliglucerase alfa or glucocerebrosidase, GC), an enzyme produced in genetically engineered carrot cells for treating type 1 Gaucher's disease (GD) by Protalix BioTherapeutics (an Israeli enterprise established in 1993) and its partner, Pfizer (Fox 2012). GD is a lysosomal storage disorder caused by a hereditary deficiency of the enzyme glucocerebrosidase, which is involved in glycolipid metabolism. GD creates a disabling condition curable only with permanent ERT.

As a result of advances in stable plant transformation technology and upstream and downstream manufacturing process, GD can be currently treated by enzyme replacement therapy using this recombinant GC that is administered intravenously every 2 weeks (Shaaltiel et al. 2007) and represents an alternative, cheaper choice with respect to the CHO-based manufacturing (i.e. the recombinant human GC Cerezyme™ -Genzyme- and (VpriV®) -Shire Pharmaceuticals-). Moreover, since expression of taliglucerase alfa is targeted to the storage vacuoles of the cell using a plant-specific sorting signal, this determines the presence of mannose sugars. In fact, GC utilized for ERT must display mannose moieties for entry into the macrophage. The expression process in the plant cell results in mannose decoration without glycan enzymatic remodelling, which is, instead, a downstream process necessary for drug of mammalian cell origin elevating the cost of goods for this specific product. Protalix brilliantly took advantage of the plant secretory pathway to obtain homogeneous paucimannosidic glycans in a cost-effective glycoengineering intervention within the cells. A fortunate circumstance to speed up ELELYSO approval was the US FDA warning about the potential for foreign-particle contamination in some Genzyme products including Cerezyme™ (Drake et al. 2017). This event has been fundamental to raise awareness of the scarcity of approved safer alternatives to mammalian cell manufacturing. Moreover, it sets a positive example to be followed that could renew investors' interest in molecular farming companies, similar to what has occurred right after commercial approval for ELELYSO that

determined a collaboration agreement Protalix BioTherapeutics-Pfizer. In addition, it should be positively noticed that 1-year treatment with taliglucerase allows patients and national sanitary systems for an estimated cost reduction by 25% with respect to administration of imiglucerase for the same period. Also the oral route of administration is currently being tested that might reduce discomfort or pain related to intravenous administration. The results demonstrate that the carrot cells protect the recombinant protein in the gastric environment and may enable absorption in vitro. Feeding rats and pigs with carrot cells containing GC proved that active recombinant enzyme can be found in the digestive tract and blood and can reach the target organs (Shaaltiel et al. 2015). These results demonstrate that the oral administration of proteins encapsulated in plant cells is feasible and may be a valuable alternative to intravenous administration of ERT.

Recombinant canine interferon-alpha (Interberry-alpha) was produced by Hokusan Co. Ltd. in the National Institute of Advanced Industrial Science and Technology (NAIST), Hokkaido, Japan. Interberry-alpha is manufactured in genetically modified strawberries in a contained biosafety level 2 facility for transgenic plants (avoidance of gene release into the environment). This product was approved for commercialization by the Japanese Ministry of Agriculture, Forestry and Fisheries, and processed strawberries containing were marketed from 2014 for the treatment of periodontal disease in dogs (Drake et al. 2017).

Production of proteinaceous drugs other than vaccines and antibodies has been proven in a variety of plant-based expression platforms (from plant cell suspension cultures to moss, to duckweed) for different applications (Table 7). The advancement status of these products reached phase I and II clinical trial and hopefully can reach commercial approval.

6 Bringing "Functional" Plants to the Marketplace

6.1 Good Manufacturing Practices

Importantly, and independently from the method used (stable/transient expression), current EMA regulatory guidelines (EMA 2008) indicate that all biopharmaceutical products intended for clinical trials should be manufactured according to GMP. Thus, GMP compliance appears to be a crucial point in developing plant-based pharmaceuticals for clinical use. In Table 8 the main GMP factories for the production of plant-made biopharmaceuticals are listed. Besides the GMP facility for the production of the only plant-made therapeutic enzyme approved for human use in Israel (Protalix/Pfizer), two big facilities are capable of biomanufacturing plant-derived pharmaceutical proteins under GMP conditions. Fraunhofer CMB in the United States deals with regulatory and clinical affairs by managing a GMP pilot plant equipped with complete processing cycles for transient expression (i.e. plant farming, bacterial cultures, plant infiltration, biomass harvest and protein purification). Also Medicago Inc., Québec CA, is into clinical trial operating in a similar facility.

Table 7 Examples of plant-based protein pharmacological entities at various stages of clinical trials

Product/application	Plant host	Status	Company (sponsor)
Taliglucerase alfa (Human glucocerebrosidase, prGCD) ERT for Gaucher's disease	*Daucus carota* (carrot) cell culture	NCT00376168 Phase III (completed in 2012) FDA-approved in 2012	Protalix BioTherapeutics, Israel/Pfizer
Moss-aGal (Human alpha-galactosidase A) ERT for Fabry disease	*Physcomitrella patens* (moss)	NCT02995993 Phase I	Greenovation Biotech GmbH, Germany
PRX-102 (Human alpha-galactosidase A) ERT for Fabry disease NCT01769001	Tobacco cell culture	Phases I and II	Protalix BioTherapeutics, Israel<aq2
Recombinant human intrinsic factor Dietary supplement for vitamin B12 deficiency	*Arabidopsis thaliana*	NCT00279552 Phase II (completed in 2006)	University in Aarhus, Denmark
Recombinant lactoferrin Anti-inflammation treatment for HIV patients	*Oryza sativa* (rice)	NCT01830595 Phase II (completed in 2006)	Jason Baker (MMRF), USA
rhLactoferrin Treatment for chronic inflammation in the elderly	Rice	NCT02968992 Phase II	Johns Hopkins University, USA
Locteron (controlled-release interferon alpha 2b) Antiviral treatment for hepatitis C virus infection	*Lemna minor* (duckweed)	NCT00593151 Phases I and II (completed in 2009)	Biolex Therapeutics, USA
Interberry-alpha Canine interferon-alpha treatment of periodontal disease in dogs	Strawberry (stable)	Manufacturing and marketing approval by the Japanese Ministry of Agriculture, Forestry and Fisheries	NIAIST Institute (Japan)

Table 8 The main GMP factories for the production of plant-made biopharmaceuticals

Company	Plant/expression system	Main biopharmaceutical product
Kentucky Bioprocessing (USA)	Potato (stable) Tobacco (transient)	Norovirus vaccine Ebola virus vaccine (ZMapp)
SIGMA Aldrich (USA)	Maize (stable)	Trypsin
Medicago Inc. (Canada)	Tobacco (transient)	Influenza vaccine
Protalix/Pfizer (Israel)	Carrot (stable)	Taliglucerase alfa
Fraunhofer (CMB-USA/IME-Germany)	Tobacco (transient, stable)	Influenza vaccine, anti-HIV antibodies
NIAIST Institute (Japan)	Strawberry (stable)	Canine interferon-alpha
Tokyo University (Japan)	Rice (stable)	Cholera toxin B subunit

6.2 Risk Analysis and Regulations

As any pharmaceutical molecules, plant-based biologics may be associated with some risks. In the particular case of plant-based pharmaceuticals, some specific features have to be considered.

It is obvious that these products should be free of classic impurities described in other recombinant product manufacturing and in the case of a biosimilar or biobetter requires demonstration of substantial equivalence to any legally marketed product. This means that the new product is at least as safe and effective as the predicate. In the area of plant-derived biopharmaceuticals, the main safety issues to be addressed could be the different post-translational modifications of the plant cell, such as the *N*-glycosylation that may induce allergic responses (mainly superable by glycoengineering of plant expression; see below).

The potential risk of impact on the environment is limited, since plant-based pharma production is strictly regulated and mainly envisaged under GAP (Good Agricultural Practices) and GMP indoor manufacturing. Transient expression systems seem to pose some risks in that, while enabling the rapid and robust production of target proteins, they require the large-scale use of *A. tumefaciens* or viral vectors, which are the only real GMO involved in the process. Therefore, agro-infiltration is covered by the existing legislation and risk assessment as GM micro-organisms in plants and therefore might not fall under the scope of the genetically modified plant legislation (Sparrow et al. 2013).

The use of CRISPR-Cas9 systems may alleviate regulatory concerns related to genetically modified plants. Beside CRISPR, also other new plant breeding techniques (NPBT) differ substantially from the transgenic techniques that have been used in the last two to three decades. For some of them, the regulatory framework in the EU may no longer be feasible. In fact, the nuclease techniques used for only point mutations or a few nucleotide changes might be considered not to determine GMO as no foreign DNA is integrated in the plant cell. NPBTs used, instead, for gene integration are covered by the existing guidance for risk assessment and GMO legislation as they do not differ from the integration of genes by transgenesis, even though greatly enhancing the accuracy of gene integration at the target locus and minimizing unattended side effects which might result in an easier risk assessment.

In the United States, the FDA ensures the safety of manufacturing (including approval of GMP facilities for plant production) and clinical use of plant-based biopharmaceuticals before licensing. The US Department of Agriculture (USDA) also plays a role in the introduction of plant-made pharmaceuticals, being responsible of the control and approval of veterinary biologics through the control of the genetic background of plants, the probability of cross-pollination and evaluating risk-management strategies.

In the European Union, the European Food Safety Authority (EFSA) and the European Medicines Agency (EMA) are in charge of the regulation concerning food safety, hence transgenic plants cultivation, and concerning medicines, respectively.

EMA published a draft guidance on the quality of the biologically active substances produced by stable transgene expression in higher plants, which only goes through plants with stable expression of transgenes and does not consider transiently transfected plants and/or plant cell cultures, the use of which still requires regulation. EMA states the importance of the establishment of master and working seed banks from the final transformant that have to be characterized in respect to the transgene (i.e. sequence, integrity, site of insertion, copy number, fate of the marker sequence), recombinant protein expression (i.e. tissue and organ specificity, regulation, expression level) and unintended changes in the levels of endogenous plant proteins and storage properties (storage conditions, shelf-life). Importantly, EMA regulatory guidelines indicate that all biopharmaceutical products in trials should be manufactured according to GMP (European Medicines Agency. Guideline on the Quality of Biological Active Substances Produced by Stable Transgene Expression in Higher Plants www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2009/09/WC500003154.pdf).

The Pharma-Planta Consortium (including 33 academic and companies in Europe and South Africa), funded by the CE Framework Programme 6, was the first entity to be engaged with regulatory authorities in this field, from 2004 to 2011. Great merit should be attributed to this consortium that significantly contributes to the maturation of the EMA guidelines in this research area (as summarized previously in the text). As a consequence, a GMP manufacturing license (i.e. for the above-mentioned anti-HIV antibody P2G12) was granted to Fraunhofer IME for plant-derived monoclonal antibodies by the Germany regulatory authority and subsequent clinical trials application approved by the UK regulatory authority. These two achievements demonstrated that a GMP-compliant process for transgenic plants and the administration of a plant-made recombinant biopharmaceutical were feasible and acceptable to regulators throughout Europe (Ma et al. 2015).

6.3 *Acceptance and Inclusion of Citizens in the Decisional Processes*

Today, the users of products are increasingly active and engaged consumers, especially when goods are relevant to their health. People are more and more aware of the consequences of health effects, and it is fundamental to be responding to people's expectations. The vast majority of consumers agree that development in science and technology could make changes to their lives too quickly and that they may hide negative side effects on both health and environment (Castle and Dalgleish 2005). Social and environmental impacts are increasingly complex, and it is necessary to demonstrate that any research is ethically, socially and environmentally responsible. Biotechnology innovations, certainly successful on technical merits, must also be accompanied by social acceptance, assuming that market demand is favourable for a

commercial success. In other words, it is a fact that benefits derived from PMP are now well highlighted; still regulatory agencies need to cooperate with developers to ensure that there is strict regulatory oversight of these products, in order to gain trust, confidence and support of the public, concerned stakeholders and investors and then progress on the marketplace.

Responsibility in science and technology is a hot topic among policy-makers, researchers and innovators in Europe. Thanks to concerted efforts, in recent years, more people now understand changes that the European research and development system is going through. More citizens are involved in science through public engagement exercises. Biology and society scientists have established collaborations. Users are leading innovation. Open access trends are changing the publishing system. The so-called responsible research and innovation (RRI; Fig. 3) represents a cross-cutting issue that is strongly pursued through the Horizon 2020 funding scheme, in line with Europe 2020 – the EU's strategy for growth launched in 2010 – and following the 2009 Lund Declaration (updated in 2015), which called upon European nations and institutions to focus research on the "grand challenges" facing society, such as climate change, water shortages and ageing populations. Innovative solutions that improve people's lives and platforms that better communicate these solutions and address ethical, social and environmental issues are the pillars of RRI (von Schomberg 2013).

If there is an area where the benefits of responsible research activities can be easily proved, it is the health sector. There is no doubt that citizens more readily welcome the results of research if they affect the quality of life. Integrating principles of responsible research and innovation in research and development activities in the field of PMPs will probably help to improve social acceptance of plant biotechnology innovations. Social actors may be motivated to participate to research and

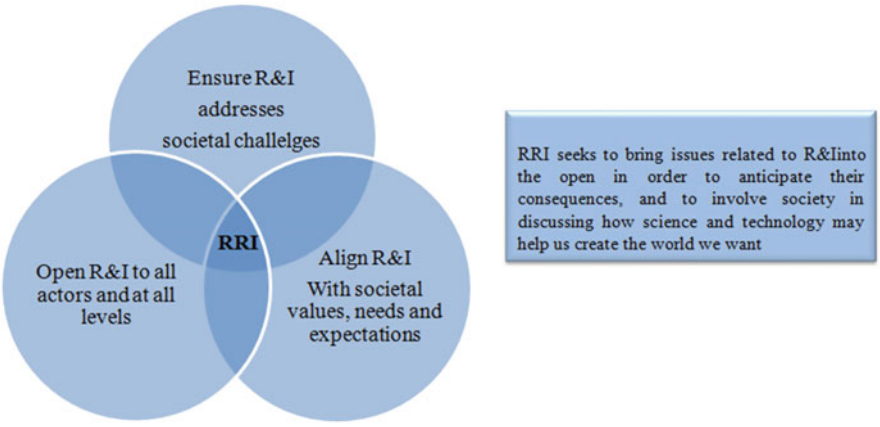

Fig. 3 Responsible research and innovation (RRI). Sourced from "A practical guide to responsible research and innovation key lessons from RRI tools" (https://www.rri-tools.eu/documents/10184/16301/RRI+Tools.+A+practical+guide+to+Responsible+Research+and+Innovation.+Key+Lessons+from+RRI+Tools)

innovation processes and may discover that outcomes of research and innovation in the field of PMPs meet the expectations of safety, desirability, acceptability and quality.

Civil society actors should be encouraged to work in contact with scientists and companies at a deeper level throughout the whole R&I (research and innovation) process. This will ensure that R&I results meet societal expectations.

If social acceptance is of sufficient importance, then gauging public attitudes toward any new technology becomes an important step in market assessment and justification of financial investment to conduct research and development. Plant-made biopharmaceuticals are on the intersection among the R&D areas of modern agriculture, medicinal plants/supplements and (plant) biotechnology (Fig. 4). The available studies show an acceptance of PMPs as a rare combination of medical and agricultural biotechnologies (Castle and Dalgleish 2005). As shown by some studies, there appears to be a positive public outlook about engineered pharmaceuticals, in general (Castle and Dalgleish 2005). The development of PMPs promises significant advantages for the production of vaccines and other therapeutic/prophylactic entities. However, the benefits and risks associated with new technologies must still be communicated and discussed with the public early, to maximize social acceptance. In fact, the above-mentioned study underlines that people are normally excluded from public debates and policy-making and normally do not have access to data and information about issues, if not through the media which most of the interviewees consider as giving misleading information. Studies have shown that even if people associate technology with relatively high risks and unknown consequences (especially genetic technologies), they still might not reject the technology (Castle and Dalgleish 2005). Oversight by regulatory agencies may give confidence to the general public and facilitate acceptance of new technologies, despite negative perceptions with regard to specific risks. More empirical research on public

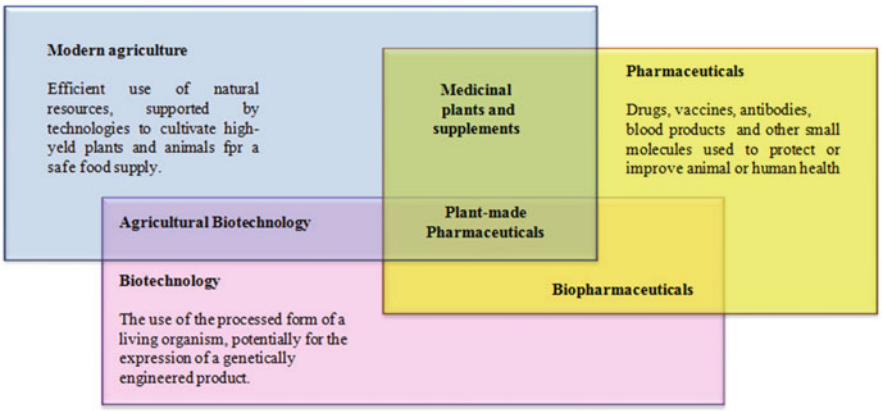

Fig. 4 Plant-made biopharmaceuticals on the intersection among the R&D areas of modern agriculture, medicinal plants/supplements and (plant) biotechnology (sourced from https://www.rri-tools.eu/it/how-to-stk-pm-implement-rri-at-national-level; R&I: Research and Innovation)

perceptions of agricultural biotechnology specific to producing novel vaccines and pharmaceuticals is needed before substantive generalizations can be made. Given that (1) oral pharmaceuticals are preferred, (2) people believe that most vaccines are genetically modified, and (3) public has expressed a high acceptance for PMPs, further development of this technology by commercial parties is desirable, if paralleled with appropriate market demand for specific products. Investment in clear communication by scientists and regulators will further enhance the public trust, optimism and ultimate acceptance for PMPs.

Even though the first players that tried to move the border forward were academia and public parties, due to the commercial uncertainties caused by production processes in the absence of certain regulations, nevertheless Elelyso is a positive example of how interest and engagement of a company can generate a big value for citizen's health and can reassure people on the possibility to get profit of some pharmaceuticals that are "biobetters by nature" due to the plant origin.

Even if the probability and severity of risks is different between open-field production for food and pharmaceutically based GMP production, depends on the plant species and has to be determined on a case-by-case basis for each plant-based product, the evidences of harmlessness of GM crops used for food supply may probably help to communicate to end-users the intrinsic safety of plant-produced biopharmaceuticals for both people and environment. As an example, meta-analysis of 21 years of open-field data (from 1996 to 2016) on the impact of genetically engineered maize clearly demonstrated the fulfilment of the desired effects on the target insect, no substantial effect on insect community diversity, reduced health risks for human consumption (due to relevant reduction of mycotoxin content in grains) and improved quality of the production (Pellegrino et al. 2018).

Reaching citizens and stakeholders with correct and peer-reviewed scientific information should finally clear both risks and benefits related to genetically engineered plants and reduce debate, hopefully. As gene technology moves forward, new traits and new health properties will be conferred to plants, and new experimental data should be available allowing researchers and regulators to draw further conclusions on the agro-environmental and health risks of GE plants.

7 Conclusions

Platforms for PMP are different and may involve the use of whole plants in greenhouses, vertical farming facilities or plant cell/organ cultures subjected to a transient or stable expression (targeted or constitutive). In addition, the unaltered version of the engineered plant may be a food or feed crop, or neither. Expression may involve transgene(s) vehiculation into nuclear or organelle genome resulting in protein monomers, multimers or virus-like particles. The PMP may be intended for purification, or administration as a crude extract or whole-plant tissue with a route of administration may be parenteral oral, nasal or topical. All these aspects may emphasize the advantages of the plant-based system but also influence the steps

toward the commercialization process. The aim of molecular farming is to produce large quantities of active and secure cost-effective pharmaceutical proteins. These approaches, in comparison with other established microbial and animal expression systems, show various advantages in the biosynthesis of pharmaceutical biomolecules. So far, lots of valuable pharmaceutical proteins and antibodies have been produced by this method, which can help the treatment of patients remarkably in developing countries where the production and preservation costs of such medicines cannot be afforded. However, there are still some disputes, such as the acceptance by the general public and the biosecurity hurdles. On the other end, it is astonishing to consider that a technology that began with the hypothesis that the plant cells could behave as a reactor for newly induced biosynthesis has actually resulted in life-saving treatments like in the case of Z-Mapp and Elelyso. The recent past has seen the emergence of PMP-based companies and the beginning of commercial era of PMP products. The success of Protalix Biotherapeutics and Medicago (just to name one of the expanding companies in the field) and many others will probably sustain the future of the PMP field. Looking ahead, the progress of further products to market and the gaining of a wider acceptance of the biopharmaceutical industry are hoped.

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