Biotechnol Lett (2015) 37:265–279
DOI 10.1007/s10529-014-1699-7

REVIEW

Recent developments in therapeutic protein expression technologies in plants

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Received: 31 July 2014 / Accepted: 6 October 2014 / Published online: 18 October 2014

Abstract Infectious diseases and cancers are some of the commonest causes of deaths throughout the world. The previous two decades have witnessed a combined endeavor across various biological sciences to address this issue in novel ways. The advent of recombinant DNA technologies has provided the tools for producing recombinant proteins that can be used as therapeutic agents. A number of expression systems have been developed for the production of pharmaceutical products. Recently, advances have been made using plants as bioreactors to produce therapeutic proteins directed against infectious diseases and cancers. This review highlights the recent progress in therapeutic protein expression in plants (stable and transient), the factors affecting heterologous protein expression, vector systems and recent developments in existing technologies and steps towards the industrial production of plant-made vaccines, antibodies, and biopharmaceuticals.

Keywords Antibodies · Bacterial cells · Biopharmaceuticals · Mammalian cells · Protein expression · Vaccines

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Introduction

The constant threat of disease-causing microorganisms is a serious concern and has evoked a paradigm shift in the pharmaceutical and biotechnological industries, prompting them to exploit the heterologous expression of compounds in living systems. Plants occupy an important position in the current short list of biofactories and promise rapid developments in the field of plant-derived biopharmaceutical agents and edible vaccines. The simple and convenient approach involved, the high yields of proteins, the lower production and storage cost, the elimination of pathogen contamination, the little processing required, and the secure delivery of oral vaccines are the predominant benefits that have boosted the use of this system in recent years. However, certain limitations often reduce the expression of target genes in plant systems, encouraging researchers to comprehensively investigate heterologous protein expression in plants and to develop novel strategies to ensure the sufficient expression of biopharmaceutical peptides that can induce immune responses.

Background

The advent of recombinant DNA technology has widened the research arena for biologists. The manipulation of *Escherichia coli* provided the first expression system (Itakura et al. 1977) for therapeutic proteins, pioneering the production of recombinant proteins. The subsequent US Food and Drug Administration (FDA) approval (Human insulin receives FDA approval 1982), of an *E. coli*-based insulin (Goeddel et al. 1979) confirmed the utility of recombinant therapeutic protein production. As a prokaryote, *E. coli* cannot accurately express complex eukaryotic protein because almost all eukaryotic proteins are modified post-translationally and require the appropriate machinery to impart the characteristic structural features that are essential for their functional integrity. Resolution of this problem has led to the development of other expression systems, including the yeast system, the *Baculovirus* system, the mammalian cell system, and the plant expression system. Each type of expression system has been used extensively for the production of recombinant therapeutic compounds, with varying success, because no single system is universally appropriate for all tasks. However, plant expression systems excel in the production of plant-derived edible vaccines (Goeddel et al. 1979; Mishra et al. 2008; Yang and Yang 2010) and have tremendous growth potential as the basis of the modern discipline, providing immediate cures for infectious diseases. Simultaneous expression of multigenes reviewed by (Zorrilla-Lopez et al. 2013) into plants can alter complex metabolic pathways that can be used to produce compounds of pharmaceutics.

The transfer of foreign DNA from diverse organisms and its integration into a host genome form the backbone of recombinant DNA technology. However, the expression of a foreign gene in a host plant cell is dependent on the cumulative effects of several elements essential for cell transformation (coding sequence, promoter region, transcript termination, etc.), the plant cell pH, the efficiency and accuracy of the transcriptional and translational machinery, plant cell biochemistry, the availability of the amino acids required for the recombinant protein, the interaction between and storage of the expressed proteins in the plant cellular environment, and many other predictable and unknown factors. Moreover, various modern plant biotechnological strategies are evolving for the targeted optimization of recombinant protein expression in host cells (including plant cells) using novel strategies (Fahad et al. 2014).

The expression of biopharmaceutical proteins in plants is based on both stable and transient expression systems. The stable transfer of a foreign gene is targeted to either the nucleus or chloroplast. The stable nuclear and plastid expression in recombinant plants (Horsch et al. 1985; Verma and Daniell 2007) and pharmaceutical proteins dates back to 1989, when transgenic-tobacco-derived immunoglobulins were efficiently assembled as functional antibodies in plants (Hiatt et al. 1989). Several studies were subsequently conducted that confirmed the use of plants to produce edible vaccines by applying this technology to rice, (Nochi et al. 2007; Shin et al. 2013; Tokuhara et al. 2013; Yuki et al. 2013), carrot (Rosales-Mendoza et al. 2008) and soybean (Moravec et al. 2007). The delivery

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of the gene of interest to the plant cell nucleus is achieved either with microprojectile bombardment (Lindbo 2007a) or, preferably, with *Agrobacterium*-mediated transformation (Nochi et al. 2007) or mediation by another non-*Agrobacterium* species (Broothaerts et al. 2005). Horizontal gene transfer and the constant expression of recombinant proteins are prominent features of stable nuclear transformation. Although this is a classical strategy, nuclear transformation is associated with several problems, including gene silencing (Chebolu and Daniell 2009), haphazard gene integration resulting in position effects of the transgene (Gorantala et al. 2011), low yields (<1 % of the total soluble protein), and a high risk of transgene contamination, which strongly discourage the commercialization of the strategy for large-scale plant-derived pharmaceutical production.

Stable plastid transformation has become an alternative strategy to nuclear transformation for the commercial production of plant-based and edible pharmaceutical compounds. Chloroplasts are characteristic plant cell organelles that are maternally inherited in most plant species (Hagemann 2004), with tiny DNA genomes of 120–150 kb (Bendich 1987). Each mesophyll plant cell contains approx. 100 chloroplasts, with 100 copies of the plastome in each chloroplast. This prompted the idea of expressing multiple transgenes with a single transgenesis process. This also extends the production potential of the transgenic plant (Maliga 2004), a crucial step in the commercialization.

An interesting feature of chloroplast DNA is that it contradicts the principles of Mendelian inheritance because it is maternally inherited in most plants, thus prohibiting gene contamination (Kittiwongwattana et al. 2007; Wang et al. 2009; Lossl and Waheed 2011). However, the pollen of transplastomic plants may occasionally be responsible for transgene contamination (Daniell et al. 1998; Svab and Maliga 2007). Therefore, rigorous selection of the parental lines is advocated by researchers to avoid this exceptional possibility (Svab and Maliga 2007; Ruf et al. 2007). Furthermore, the presence of a single promoter is sufficient to express multiple genes because most chloroplast genes are arranged in operons and are transcribed as polycistronic mRNA (Quesada-Vargas et al. 2005). In short, the potential for polycistronic expression, with minimal chance of horizontal or vertical gene transfer through pollen transmission, the enormous replication potential, and very high yields of proteins in transplastomic plants are the key benefits of plastid transformation over nuclear transformation.

The transient expression of foreign genes in plants does not require the transgene to be integrated into the host genome nor does it follow the molecular central dogma for expression in plants. However, it provides an opportunity to express recombinant proteins within a very short period of time (Verma and Daniell 2007), another novel attribute that has stimulated the development of plants as biofactories for pharmaceutical production. Therefore, the rapid commercialization of plant-derived vaccines, antigens, antibodies, and therapeutic drugs is likely in the near future based on the distinct merits of this transient expression system, including its rapid expression of target genes, the simplicity of the system, the fact that it does not require sophisticated laboratory equipment or techniques, and the possibility of improving expression levels with novel optimization techniques (Komarova et al. 2010).

The first in vitro and in vivo expression of viral RNAs (Marillonnet et al. 2004) were milestones in this field and encouraged researchers to explore the utility of plant viral vectors for producing recombinant proteins in crop plants. Plant viral vectors do not integrate into the host genome and are characterized by their tremendous expression potential because they are rapidly transmitted from one plant cell to another (Tiwari et al. 2009). The expression of an antigen epitope (Haynes et al. 1986) was demonstrated using the tobacco mosaic virus (TMV)-coat protein in a plant system. A short comparison of the technologies used in the production of recombinant proteins as biopharmaceutical agents is given in Table 1.

Plant system versus other heterologous protein expression systems

Pharmaceutics proteins, such as hGAD65, NVCP, 2G12 and hIL-6, have been produced using heterologous systems, for example in bacteria, yeast, mouse embryo cells, *Spodoptera frugiperda* cells, baby hamster kidney cells and hybridoma clones with different level of expressions. The same recombinant proteins have been expressed and produced in photosynthetically-active organisms, such as plants and *Chlamydomonas reinhardtii* using different

Table 1 Comparison of stable (nuclear, chloroplast) and transient expression systems

Type of transformation	Host plant/location	Vector/promoter	Recombinant protein	Expression level	Reference
Stable expression					
Nuclear transformation	Rice endosperm	Tapur promoter	Human lysozyme	41.6 µg/grain	Hennegan et al. (2005)
	Tobacco cell compartments	pTRA	Haemagglutinin surface protein	640–1,440 mg/kg	Mortimer et al. (2012)
	Rice Seeds	CaMV-35S	Cholera toxin B (CTB)	0.3 %	Gunn et al. (2012)
	Tobacco	CaMV-35S	Heat labile enterotoxin B	1.6 %	Larsen and Curtis (2012)
	Soybean	*ubi3*	Heat labile enterotoxin B	>2.0 %	Moravec et al. (2007)
	Carrot	CaMV-35S	Heat labile enterotoxin B	3.0 µg/g	Rosales-Mendoza et al. (2008)
Chloroplast transformation	Tobacco leaves	rice psbA promoter-E2	pE2 polypeptide	pE2 polypeptide	Zhou et al. (2006)
	Tobacco	NEP promoter	HPV 16-L1 capsomere	1.5 %	Waheed et al. (2012)
	Tobacco	psbA promoter	HCV core Protein	0.1 % TLP	Hiroi and Takaiwa (2006)
	Tobacco	prrn promoter	C4V3 protein	25 µg/g FW	Tregoning et al. (2005)
	Sugarbeet	prrn promoter	GFP	NA	Haq et al. (1995)
	Brassica	16S rRNA promoter	Anti-spectinomycin	NA	Turpen et al. (1995)
Transient expression					
Epitope presentation	Tobacco	CMVCP–F/HN	CMV VLPs	1–2 mg/ml	(Rigano et al. 2013)
	Tobacco	Potato virus X	HPV-16 L2	170 mg/kg	McCormick et al. (2008)
	Tobacco	Potato virus X Alt. mosaic virus	Influenza virus M2E	1–3 mg/g	Mason et al. (1998)
Agroinfilteration	Tobacco	Binary vector	GFP/HFBI	38 %	Itakura et al. (1977)
	Tobacco	Ptrac	HIV-1 pr55GAG	0.3 %	Pillai and Panchagnula (2001)
	Tobacco	Binary vector	HIV monoclonal antibody 2G12	NA	Mishra et al. (2008)
	Tomato	Pepino mosaic virus PepMV	FMDV 2A catalytic peptide	0.2–0.4 g/kg	Boothe et al. (2010)
magniCON	Tobacco	PVX amplicon vector	COPV L1-protein	NA	Azhakanandam et al. (2007)
	Tobacco	TRBO	GFP, HA peptide	3.3–5.5 g/kg	Lindbo (2007a)
	Tobacco	TMV vector	GFP	NA	Lindbo (2007b)
	Tobacco	pTBSV	HBc (VLPs), GFP	0.8 mg/g FW	Huang et al. (2009)
	Tobacco	TMV-Gate vector	GFP, GUS	2.5–4.7 mg/g	Kagale et al. (2012)
Suspension cell culture	Tobacco	CaMV-35S	Active dust mite allergens	NA	Lindbo (2007b)
	Tobacco	CaMV-35S	GUS	0.03–0.12 %	Broothaerts et al. (2005)

Table 1 continued

Type of transformation	Host plant/location	Vector/promoter	Recombinant protein	Expression level	Reference
Targeted specific expression	Tomato fruit	Pepino mosaic virus PepMV	FMDV 2A catalytic peptide	0.2–0.4 g/kg	Sempere et al. (2011)
	Rice seeds	CaMV-35S	Cholera toxin B (CTB)	0.3 %	Gunn et al. (2012)
	Rice endosperm	Tapur promoter	Human lysozyme	41.6 μg/grain	Hennegan et al. (2005)
Virus induced gene silencing (VIGS)	Soyabean	Apple latent spherical V irus (ALSV) vector	–	Transmission of silencing (20–30 %)	Yamagishi and Yoshikawa (2009), Sasaki et al. (2011)
	Wheat spike	Barley stripe mosaic virus (BSMV) vector	NA	NA	Ma et al. (2012a, b)
Viral like particles (VLPs)	Tobacco	Binary vector pPSP19	Hepatitis B core antigen	0.8 mg/g FW	(Huang et al. 2009)
	Tobacco	Chimeric double CaMV-35S promoter	HA peptide	50 mg/kg FW	D'Aoust et al. (2008), Landry et al. (2010)
Codon optimization	Tobacco	pZP200 with double CaMV-35S promoter	SAG1 protein	1.3 μg/g FW	Laguía-Becher et al. (2010)
3′ and 5′ UTRs	Tobacco/cotton	CaMV-35S promoter with 28nt synthetic 5′UTR	GUS protein	2.7–14,955.0 pmol MU/min/μg protein	Kanoria and Burma (2012)
	Arabidopsis	CaMV-35S promoter with Fluc mRNA attached to 5′UTR	HPR (By2)	23 mg/L	Matsui et al. (2012)
	Tobacco	T7 promoter with attached bacteriphage 5′UTR	Ce16a/aadA	10 % TSP	Yang et al. (2013)
Epigenetic modifications	Tobacco	Tomato leaf curl Java begmovirus vector	βC1/GFP	Silenced expression observed	Kon et al. (2007)
	Tobacco	Binary vector (PVX and TRV vectors)	GFP	Silenced Expression observed	Buchmann et al. (2009)

Table 2 Comparison of different systems used for the expression of heterologous proteins

Expression system	Major advantages	Major limitations	Approved biopharmaceutics
Bacterial cell system (*E. coli*)	Availability in short duration and the most simplest system	Unable to perform glycosylation in recombinant proteins	ABthrax (Rader 2013) Human genome sciences ins., GSK
Yeast cell system	Correct folding in functional recombinant proteins, low cost of purification	Produced sialylated glycoproteins are not found fit for human consumption	Recombivax HB (Gilbert et al. 2012) MERCK KGAA
Baculovirus expression vector system (BEV)	Able to produce glycosylated recombinant proteins and suited for production of proteins requiring post-translational modifications	Presence of lipidic envelopes in virions and less efficient in processing of polyproteins	Flublok (Treanor et al. 2011) Protein Sciences Co.
Mammalian cell system	Highly adaptive and able to produce glycosylated protein with post-translational modifications	Associated with slow growth, increased fermentation cost and higher risks of viral infection	Flucelvax (Rader 2013) (Novartis)
Plant cell system	Rapid growth, low cost of purification, highly adaptive for producing glycosylated protein and any modification in Expression system is possible	Highly specific to plant of choice and any universal recombinant production system have not reported to date	Recombinant (Rader 2013) Glucocerebrosidase (Protalix)

organs of plants like leaves, seeds, tubers and tape roots (Merlin et al. 2014). Plants maintain a strong position among the heterologous protein production biofactories, with many advantages over other systems. The important benefits of using plants as biofactories include their ease of growth, their relatively low water usage, lower storage costs, requirements for only CO_2 and minerals to grow, their adaptability to cell culture or agricultural production, the lack of pathogen contamination, and a highly scalable production system. Plants also post-translationally modify the expressed proteins correctly, allowing their proper functioning (Lossl and Waheed 2011). *Escherichia coli* is inappropriate for the expression of some antigenic proteins because it lacks the capacity for a variety of post-translational modifications and folding requirements. Yeast and insect cell lines can perform some of these essential post-translational modifications but there can be immunologically-significant differences in these modifications, which limit the usefulness of these systems as expression platforms for vaccine development (Houdebine 2009). In Table 2, the different expression systems used to produce heterologous proteins are compared and their most notable advantages and limitations specified.

Plants as heterologous protein expression systems to fight infectious diseases and cancers

The important viral diseases that cause significant deaths or pandemics in human populations are influenza, measles, hepatitis B, hepatitis C, hepatitis E, human immunodeficiency virus acquired immunodeficiency syndrome (HIV-AIDS), human papilloma virus (HPV) infection, and rabies, whereas considerable economic losses in animals are attributable to avian influenza, Norwalk virus, and foot and mouth disease. Cholera, tuberculosis, and diphtheria are among the bacterial diseases that cause considerable loss of life. Plants are used as expression systems to produce vaccines and other pharmaceuticals used as prophylactic and curative agents for these diseases. Some biopharmaceuticals recently produced in plants against important viral, bacterial, and protozoan diseases and cancers are listed in Table 3.

Strategies to enhance transient recombinant protein expression in plants

Several plant transient expression rely on viral vector systems. In the 1990s scientists were using

Table 3 Biopharmaceutical compounds developed against infectious diseases and cancers using plants as biofactories

Disease	Pathogen	Bio-pharma-ceutics	Promoter/vector	Expression system	Reference
Protozoan infection					
Malaria	Plasmodium	$pyMSP1_{19}$	Deconstructed TMV vector	magnICON Tobacco	Ma et al. (2012a, b)
		CTB-MSP1 AMA-1	psba/rrn Promoters	Transplastomic transformation lettuce/choloroplast	Davoodi-Semiromi et al. (2010)
Bacterial diseases					
Cholera	*Vibrio cholarae*	CTB	TMV vector	magnICON tobacco	Hiatt et al. (1989)
		LTB	CaMV-35S Codon optimized carrot	Somatic embryogenesis	Kohl et al. (2007), Mason et al. (1992)
		LTB-ST	prrn promoter	Transplastomic transformation tobacco	Koya et al. (2005)
		Seed-specific LTB	Soyabean glycinin promoter	Stable transformation somatic embryogenesis soyabean	Gleba et al. (2005)
		CTB-MSP1 AMA-1	Psba/rrn promoters	Transplastomic transformation lettuce/chloroplast	Laanger (2011)
Tuberculosis	*Mycobacterium tuberculosis*	Immune-dominant antigens	Patatin promoter	Stable transformation Tobacco	(Modelska et al. 1998)
		CTB-ESTA6	psbA promoter	Transplastomic transformation tobacco/lettuce	Hiroi and Takaiwa (2006)
		TB vaccine protein	CaMV-35S promoter	Agroinfilteration tobacco	Tregoning et al. (2005)
Diphtheria Pertussis and tetani (DPT)	Diphtheria Pertussis and tetani (DPT)	sDPT polypeptide	CaMV-35S	Stable transformation tomato	Soria-Guerra et al. (2007, 2011)
Viral diseases					
SARS	Corana virus (human)	SARS-Cov	OCS_3MAS	Stable nuclear transformation cauliflower	McCormick et al. (2008)
Small pox	Variola virus (human)	Viral coat B5	CaMV-35S	Stable nuclear transformation, collard	Pogrebnyak et al. (2006)
		Candidate pB5	CaMV-35S	Magnifection, tobacco	Mason et al.(1998), Itakura et al. (1977)
Diarrhea	Rota virus gastroenteritis (Human)	RV VLPs	CaMV-35S	Stable nuclear transformation, tobacco	Yang et al. (2011)
Post weaning diarrhea (PWD)	Procrine epidemic diarrhea virus (PEDV) (Pigs)	sLTB–sCOE	HMW–GS (Bx17)	Epitope presentation rice endosperms	Lindbo (2007a)
		Functional recombinant FaeG	psbA promoter	Biolistic chloroplast transformation	Kolotilin et al. (2012)

Table 3 continued

Disease	Pathogen	Bio-pharma-ceutics	Promoter/vector	Expression system	Reference
Measles	Measles virus (human)	MV-H protein	CaMV-35S	Stable nuclear transformation, tobacco	Yang and Yang (2010), Verma and Daniell (2007)
Rabies	Rabies virus (human)	Rabies nucleoprotein	CaMV-35S	Nuclear transformation and agroinfilteration, tomato	Hennegan et al. (2005)
Influenza	H1N1	HA1-protein	Binary vector AscI–PacI	Stable transformation tobacco	Nochi et al. (2007)
	H1N5	HA1-5 (VLPs)	Alfalfa plastocyanin promoter	Agroinfilteration tobacco	Landry et al. (2010), D'Aoust et al. (2008)
	H7N7	HA7-7	CaMV-35S	Agroinfilteration Tobacco	Kanagarajan et al. (2012)
	H1N5, H1N5	HA1-5/1	Launch vector	magnICON Tobacco	Chichester et al. (2012), Shoji et al. (2011)
Hepatitis	HBV	S-HBsAg	CaMV-35S	Stable transformation lettuce	Pniewski et al. (2011)
	HCV	Chimeric CMVs	**C**ucumber mosaic Virus	magnICON Tobacco	Nuzzaci et al. (2007, 2009, 2010)
	HEV	pE2	Rice-psbA promoter E2	Biolistic chloroplasted transformation tobacco	Zhou et al. (2006)
AIDS	Human immune deficiency virus HIV	C4V3 polypeptide	prrn promoter	Biolistic chloroplasted transformation tobacco	Rubio-Infante et al. (2012)
		HIVmAbs	CaMV35-S	Agroinfilteration Tobacco	Rosenberg et al. (2012)
Cancer					
Cancer	Human pappilloma virus (HPV)	HPV16-L2 epitope	PVX	magnICON, tobacco	Cerovska et al. (2012)
	Non-Hodgkin's lymphoma	HPV16-L1mAbs	CaMV-35S	Stable transformation Tobacco	Liu et al. (2013)
		HPV11-L1-NLS proteins	CaMV-35S	Stable transformation arabidopsis/ tobacco	Kohl et al. (2007)
Dengue	Dengue virus (DENV)	Dengue virus tetra-epitope peptide (cE-DI/IIp)	Tobacco psbA promoter/ pRL1001	Lettuce plastid transformation	Maldaner et al. (2013)

Agrobacterium to do transient expression in plants which does not rely on viral vectors (Kapila et al. 1997). The study of plant viruses revealed their latent ability to carry foreign genes. The discovery of the positive-sense RNA viruses, TMV, Tobacco rattle virus (TRV), and Potato virus X (PVX), facilitated their use as heterologous protein expression vectors (Hefferon 2012).

Viral vectors can be classified in different ways, according to the purpose they serve. The two main groups are (i) independent viral vectors; and (ii) minimal viral vectors. Independent viral vectors can replicate and be inoculated into plants as viral particles, multiply at the site of infection, and then move systemically as virus-encoded particles to infect maximum plant tissues. Minimal vectors, in contrast, can replicate but lack systemic movement and are modified to achieve greater protein expression. Although the discovery of plant viruses that can be used as vectors was a milestone in the production of recombinant proteins, their inability to carry large constructs hindered their development until they were optimized with much needed modifications. This limitation was addressed by the development of "magnifection" technology (Gleba et al. 2005), in which *Agrobacterium* is used as a systemic movement agent to deliver viral replicons in plants to produce high yields of recombinant proteins. This strategy combines the benefits of three systems: the DNA delivery capacity of *Agrobacterium*, the expression levels of RNA viruses, and the post-translational modifications and low production costs of plants.

Magnifection technology has many benefits, including the ease of biocontainment of the transgenes, simple scale-up, high-level expression of heterologous proteins, low cost, and versatile protein expression (single-chain antibodies, antigens, enzymes, etc.). However, it is still limited in its capacity to post-translationally modify the recombinant proteins. In particular, aberrant glycosylation patterns can make the recombinant protein nonfunctional, by affecting its immunogenicity in the case of vaccines (Gleba et al. 2005). The high expression levels of some recombinant proteins can also have a lethal effect on plants, such as the hepatitis B virus (HBV) surface antigen (HBsAg; Gleba et al. 2005) mostly on cell expansion and cell division. The production of immunoglobulin G (IgG) antibodies with magnifection is also difficult because it requires the manipulation of viral vectors. Recent modifications have generated magnICON (the trade name for magnifection), which has allowed the expression of many important biopharmaceutical products for important diseases, including pyMSP119 for malaria, using deconstructed viral vectors (Ma et al. 2012a, b). A few examples in which the magnICON system or its modified form has been used include the production of follicular non-Hodgkin's lymphoma Yusibov et al. (2011) and *E. coli* heat-labile enterotoxin B (LTB) (Rosales-Mendoza et al. 2008). Virus-like particle development is another important technique for high protein expression for example chimeric cucumber mosaic viruses (CMVs) for hepatitis C virus (Nuzzaci et al. 2007, 2009, 2010), while PVX is used in expressing HPV16-L2 against HPV (Cerovska et al. 2012).

The shortcomings of the magnICON system have been addressed by constructing the pEAQ vector system. pEAQ is a special type of non-replicating vector based on the Cowpea mosaic virus (CPMV). This system provides high recombinant protein expression without the fear of biocontamination or genetic drift (Peyret and Lomonossoff 2013). The new expression system, based on a deleted version of CPMV RNA-2 with a mutated 5′-untranslated region (UTR), enhances the expression of green fluorescent protein (GFP), DsRed, the HBV core antigen (HBcAg), and human anti-HIV antibody 2G12 (Sainsbury and Lomonossoff 2008; Joensuu et al. 2009).

To enhance heterologous protein production in plants, scientists have modified the strategies by using expression vectors derived from virus origins and utilizing reporter genes such as GUS and GFP while including plant-based introns for proper expression in eukaryotic cells (Canizares et al. 2005; Lico et al. 2008; Marillonnet et al. 2004, 2005). A minimal PVX vector solely with its RNA polymerase gene proved to be more effective by the expression of GUS protein yield which was 6.6-fold more than utilizing the full length PVX vector (Larsen and Curtis 2012). Post-transcriptional gene silencing was expressed when minimal PVX fragment was co-expressed with other solanaceae based viral vectors such as P19 (viral protein of tomato bushy stunt virus) and HC-Pro (viral protein of tobacco etch). Furthermore, enhanced expression of protein was attained while using major sequence from CPMV in non replicating viral vector (Canizares et al. 2005). Using hairy root as protein

expression system, TRV vector exhibited higher expression of protein accumulation than PVX based vector (Larsen and Curtis 2012).

A number of strategies have been used to introduce foreign genes with CMV-based systems (CMV-based inducible vectors and CMV-based advanced replicating vectors), including the manipulation of the cloning sites, which are easy to use, and also the reassortment of genotypes is taken into consideration. The deletion of the CMV movement protein also leads to greater protein accumulation. *Nicotiana benthamiana* is the most suitable host for recombinant protein production using agroinfiltration and, because of its wide host range, CMV has a particular edge as the vector of choice because various plants can be used as recombinant protein factories (Hwang et al. 2012). The hydrophobin (HFB1) sequence from *Trichoderma reesei* fused to GFP, infiltrated on *Agrobacterium*, and transiently expressed in *N. benthamiana*, was reported to enhance the accumulation of GFP, with the concentration of the fusion protein reaching 51 % of the total soluble protein, with delayed necrosis of the infiltrated leaves (Joensuu et al. 2009).

Interestingly, the GFP–HFB1 fusion was targeted to the endoplasmic reticulum (ER), where it produced large novel protein bodies. This allowed the recovery of the HFB1 fusion protein from extracts with a simple and scalable recovery process based on an aqueous two-phase system. Single-step phase separation selectively recovered 91 % of the GFP–HFB1, accounting for 10 mg/ml. Fusion with HFB1 not only increases the expression of recombinant proteins but also provides an easy method for their subsequent purification. This HFB1 fusion technology, combined with the speed and post-translational modification capacities of plants, has increased the value of transient plant-based systems (Joensuu et al. 2009). Furthermore, the lower expression of desired genes are challenged by weak promoters in plants, which can be optimized by generation of synthetic promoters as reviewed by Liu et al. (2013). The technologies described by Liu et al. (2013) can be further utilized for improving therapeutic protein expression in plants to achieve desired results.

The development of transient expression was further improved by the molecular and bioinformatic analysis of the sequences which enabled manipulation of synthetic enhancers, suppressors transcription factor binding domains and promoters. (Sainsbury and Lomonossoff 2014). These studies suggest that there is still much to be done to improve the expression of heterologous proteins in plants, most of which will involve optimizing the vector systems. It will be necessary to find regions in vector systems that do not affect their innate ability to replicate, ways to suppress transgene silencing, an interesting review by Alba et al., covers several gene silencing pathways in plants (Martínez de Alba et al. 2013) and further improvements to non-replicating viral systems that rely on hypertranslation rather than replication, such as the pEAQ vector system.

Key events in the development of the plant-derived biopharmaceutical industry

The key events in the development of plant-derived biopharmaceuticals are summarized briefly in Fig. 1. The momentum to use plants as biofactories increased when the first vaccine-related protein was expressed in transgenic tobacco plants (Hiatt et al. 1989). In the decade after this development, a number of breakthroughs occurred, in particular the expression of HbsAg in tobacco plants (Mason et al. 1992), followed by the presentation of a malarial parasite epitope (Turpen et al. 1995). Heat-labile enterotoxin B, LTB, produced in potato plants (Haq et al. 1995) as functional as that expressed in *E. coli* and the first human phase I clinical trial of plant-derived LTB then paved the way for the design and production of edible vaccines (Rigano et al. 2013).

The low-level expression of the recombinant proteins has hindered the development in this exciting field until an anthrax antigen was expressed in the chloroplast-based system and was successfully used to immunize mice (Koya et al. 2005). It was at this time that the magnifection technology was introduced to enhance heterologous protein expression (Gleba et al. 2005). This technology is an important breakthrough in increasing recombinant protein expression, with several modifications being reported subsequently. In the same year (2005), a single intranasal dose of a plant-derived vaccine produced in tobacco efficiently activated $CD4^+$ T cells and antibodies against tetanus toxin in mice (Tregoning et al. 2005).

Progress in this field continues with reports of plant-based epitope presentation of cottontail rabbit papilloma virus CRPV-L1 and the immunization of

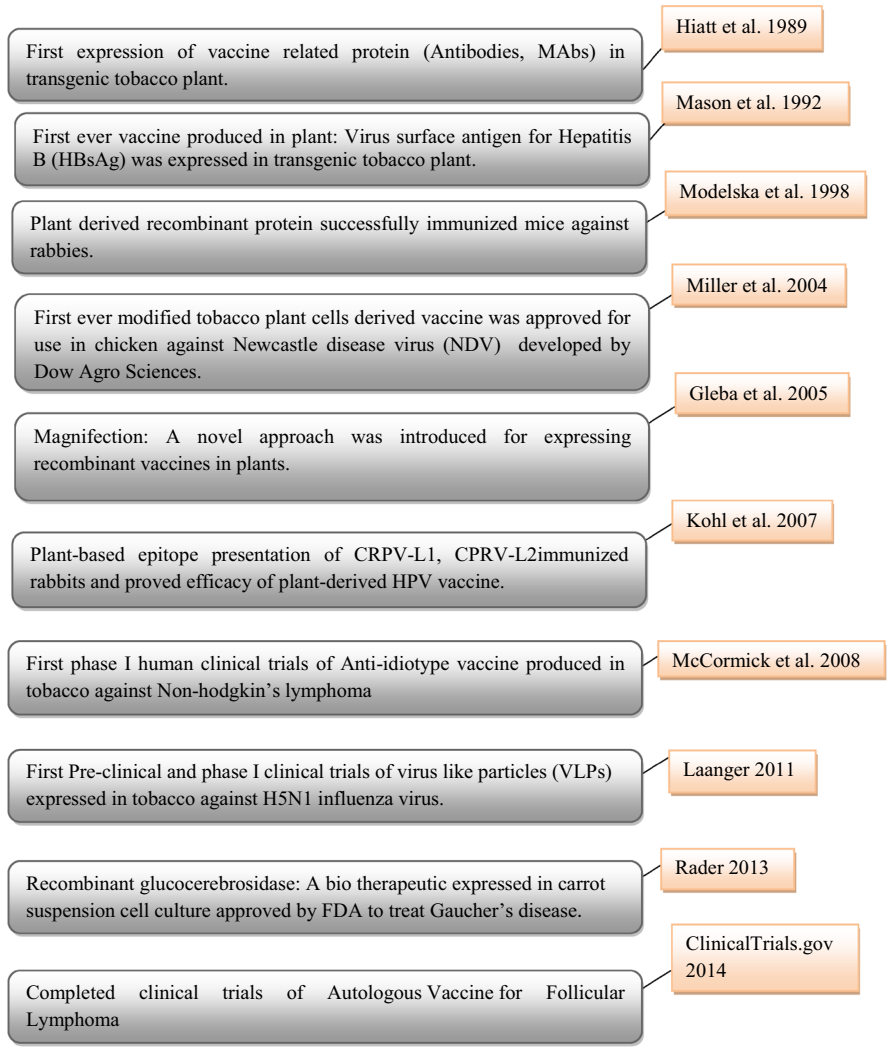

Fig. 1 Key events in the development of plant-derived biopharmaceuticals

rabbits with CPRV-L2, confirming the efficacy of a plant-derived HPV vaccine (Kohl et al. 2007). Later, the first plant-derived vaccine was approved to immunize chickens against newcastle disease virus (Miller et al. 2004), and the first phase I and II clinical trials of a plant-derived therapeutic compound from a suspension culture of carrot cells, directed against Gaucher's disease, were undertaken (Rigano et al. 2013). In 2008, the first phase I human clinical trial of an anti-idiotype vaccine against non-Hodgkin's lymphoma was performed (McCormick et al. 2008) and, in 2010, the first preclinical and clinical trials of virus-like particles (VLPs) against H5N1 influenza and the first phase II clinical trials of caroRX (a plant-derived antibody) against dental decay were undertaken (Rigano et al. 2013). In 2011, the FDA approved a phase II human clinical trial of VLPs against H5N1 (Laanger 2011).

Conclusion

Plants can provide vaccines and other therapeutic compounds in a number of ways, including in cell or root cultures, in greenhouses, or in the field. The low productivity of heterologous proteins hindered the commercialization of plant-made biopharmaceutical products for a long time but recent developments that have increased heterologous protein expression in plants with various novel techniques, including

magnifection and its optimization, have made this commercialization possible. Plant viral vectors, combined with HFB1s, provide a new way to increase recombinant protein production and to improve bioprocessing. A number of factors must be considered during recombinant protein expression, such as codon optimization, organelle- and organ-specific expression, proteases, etc., which have been extensively reviewed elsewhere. The world is threatened again by an influenza pandemic and, in such situations, plants provide a quick and reliable vaccine production system. The major hurdle remains the glycosylation pathway in plants, which is highly resistant to change, so the post-transcriptional modification of recombinant proteins for human and animals remains limited. This can be overcome by installing a novel glycosylation pathway in plants. The installation of such a novel pathway has been achieved in *Arabidopsis thaliana*, where a photorespiration suppression pathway was installed to increase the biomass production. Such novel technologies can be used to overcome health concerns by providing cheaper medicines to third-world countries, where the disease burden is high. Therefore, it is time for governments and commercial enterprises to allocate more funds for research into plant-made vaccines and therapeutic agents and their further commercialization.

Acknowledgments We thank the Key Technology Program R & D of China (project no. 2012BAD04B12) and MOA Special Fund for Agro-Scientific Research in the Public Interest of China (no. 201103003) for funding.

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