Research review paper

Tobacco, a highly efficient green bioreactor for production of therapeutic proteins

Reynald Tremblay [a], David Wang [b], Anthony M. Jevnikar [c], Shengwu Ma [a,c,d,]*

[a] Department of Biology, University of Western Ontario, London, Ontario, Canada N6A 5B7
[b] Department of Molecular Biology, Princeton University, Princeton, NJ 08544-1014, USA
[c] Transplantation Immunology Group, Lawson Health Research Institute, London, Ontario, Canada N6A 4G5
[d] Plantigen Inc., 700 Collip Circle, London, Ontario, Canada N6G 4X8

ABSTRACT

Molecular farming of pharmaceuticals in plants has the potential to provide almost unlimited amounts of recombinant proteins for use in disease diagnosis, prevention or treatment. Tobacco has been and will continue to be a major crop for molecular farming and offers several practical advantages over other crops. It produces significant leaf biomass, has high soluble protein content and is a non-food crop, minimizing the risk of food-chain contamination. This, combined with its flexibility and highly-efficient genetic transformation/regeneration, has made tobacco particularly well suited for plant-based production of biopharmaceutical products. The goal of this review is to provide an update on the use of tobacco for molecular farming of biopharmaceuticals as well the technologies developed to enhance protein production/purification/efficacy. We show that tobacco is a robust biological reactor with a multitude of applications and may hold the key to success in plant molecular farming.

© 2009 Elsevier Inc. All rights reserved.

Contents

1. Introduction

The worldwide demand for recombinant proteins is growing faster than traditional systems can keep pace. This includes valuable pharmaceutical proteins such as antibodies and vaccines as well as industrial enzymes and secondary metabolites. Traditional recombinant production systems such as bacterial and mammalian cell culture are limited in their scalability and production cost, due in part to requirement for complicated fermentation equipment and expensive downstream processing. Recombinant protein production in plants, on the other hand, offers a solution to the rising demand and provides opportunities that are not feasible with other systems.

Plants offer several advantages as "Green Bioreactors". First is the ability to perform eukaryotic post-translational modifications such as glycosylation and disulfide bridging that are often essential for biological activity of many mammalian proteins (Ma et al., 2003;

Horn et al., 2004). Green bioreactors do not suffer the same risk of pathogen contamination as seen in mammalian cell culture as there are no known cross-kingdom pathogens. Plant growth requirements are simple and inexpensive compared to traditional cell culture systems, allowing for inexpensive and nearly unlimited scalability. In cases where plant cell culture is used, it has much more simple growth requirements than mammalian or insect cell culture and is able to utilize light as its main energy source, further reducing costs. Additionally, plant systems are robust and inert, allowing for simplified handling/purification and the ability in the case of pharmaceutically relevant proteins to be administered orally with minimal processing. For the establishment of a green bioreactor, there are seemingly many plant species to choose from, each with its own advantages and disadvantages depending on the type of application desired.

Tobacco (*Nicotiana tabacum*) has been and will continue to be a major platform for green bioreactors. Despite a traditionally negative view due to its strong ties to smoking, tobacco offers several unique advantages over other plant species. Tobacco is often referred as the "white mouse" of the plant world, as it is amicable to genetic modification and has become the primary vehicle for proof-of-concept work in recombinant protein production for the last 20 years. Tobacco is a leafy plant, having high biomass yield (up to 100 t of leaf biomass per hectare) and high soluble protein levels compared with many other model and crop species, an attractive feature for a protein production platform. Tobacco also offers various ways of expressing proteins of interest, such as transient based expression via agrobacterium or viral induction and stable nuclear or chloroplastic genome based expression. The availability of low-nicotine low-alkaloid tobacco varieties such as cultivar "81V9" has made tobacco plants even suitable for direct oral delivery of recombinant antigens in plant material or crude protein extracts (Menassa et al., 2001). Tobacco is neither a food nor feed crop, thus reducing the likelihood of transgenic material contaminating the food or feed chains. These features have made tobacco particularly well suited for plant-based production of biopharmaceutical products. Indeed, the number of therapeutic proteins produced in transgenic tobacco plants is increasing steadily, with several tobacco-derived products having advanced to human clinical trials. The goal of this review is to discuss the application and potential of tobacco as a green bioreactor for recombinant therapeutic protein production.

2. Production of pharmaceutical proteins in tobacco

Tobacco is proving to be an attractive bioreactor for the production of pharmaceutically relevant proteins. In addition to the economical advantages of tobacco bioreactors, it has the ability to produce a wide range of therapeutic proteins including antibodies, vaccines and immunomodulatory molecules such as cytokines. Moreover, by using transient protein expression, tobacco is able to generate significant quantities of protein in a short period of time that is necessary for rapid response to disease outbreaks, such as the recent influenza A/H1N1 pandemic, and for the patient-specific treatment of cancer.

2.1. Antibody production

Antibodies represent the single largest class of new drug entities under development at this time. The generation of antibodies for pharmaceutical use requires the coordinated creation of both a heavy and light chain and the correct assembly into a single functional unit. Monoclonal antibodies are traditionally generated using hybridoma cell lines, which are obtained by fusion of an immortalized cancer cell with an antibody-producing splenic cell isolated from antigen-challenged host. This process is technically challenging and the growth requirements for maintaining hybridoma cells are expensive. Bacterial production is unfeasible due to its inability to glycosylate and assemble functional antibodies. Plants offer an inexpensive alternative that is able to produce authentic antibodies. Tobacco was the first host chosen to express a functional recombinant full-length monoclonal antibody (mAb), the anti-mouse catalytic IgG$_1$(6D4), in plants (Hiatt et al., 1989). It was shown that when individual tobacco plants expressing single heavy (γ) or light (κ) chains of 6D4 were generated, simple sexually crossing of two parental transgenic tobacco lines resulted in progeny lines that express and accumulate a fully assembled and functional mouse antibody at high levels (1.3% of total soluble protein (TSP)). Since then, the expression of recombinant antibodies using tobacco as a platform has been extended to include monoclonal antibodies with more complex structures such as secretory IgA, single-chain antibodies and single-chain antibody fragments of different specificity.

Secretory IgA (sIgA) is a complex, multimeric protein composed of two IgA units (2 heavy and 2 light chains), a joining J chain, and a secretory component (Johansen et al., 1999), and is the antibody naturally produced by the body to protect oral and other mucosal surfaces against infectious organisms and toxins. It is generated by a unique cooperation between two distinct cell types: J chain-expressing plasma cells that produce polymeric (p)IgA (mainly dimers), and secretory epithelial cells that express the secretory component (SC) (Johansen et al., 1999). The recombinant production of SIgA in mammalian cells is difficult and expensive, requiring two independent and mixed cell lines and constant monitoring and cell rebalancing. However, tobacco greatly reduces the complexity for its recombinant production. The best example is the tobacco-based production of a humanized secretory murine IgA1 (Guy's 13 SIgA-G). The murine monoclonal antibody IgG1 (Guy's 13) which specifically recognizes the SAI/II protein of *Streptococcus mutans*, the main causative agent for dental caries, has been used successfully to prevent *S. mutans* colonization and the development of dental caries in non-human primates as well as in human clinical trials (Lehner et al., 1985; Ma et al., 1989). Therefore, Guy's 13 IgG antibody has the potential to treat and/or prevent dental caries. To produce recombinant Guy's 13 SIgA-G, transgenic tobacco plants were generated to express independently either the Guy's 13 kappa (light) chain, the hybrid IgA-G antibody heavy chain, murine J chain, or rabbit secretory component (SC). Through a series of sexual crosses between these plants, researchers produced transgenic tobacco plants expressing a functional, high molecular weight secretory murine immunoglobulin (Guy's 13 SIgA-G), with accumulation levels up to 500 μg per gram leaf material (Ma et al., 1995). Human clinical trials showed that plant-derived SIgA/G antibody prevented oral colonisation by *S. mutans* (Ma et al., 1998). Renamed CaroRx™, the tobacco-derived SIgA/G protein became the first plant-made antibody approved for human use in 2005 in the European Union.

Tobacco has also proven to be an efficient system for generating fragments of the antibody, including single-chain variable fragment (scFv), single-chain antibody variable domain (Fv) fragment and antibody-binding (Fab) fragment (see Table 1). These small recombinant synthetic antibodies retain full antigen-binding activity but lack specific assembly requirements. They are being used in diagnosis and treatment (Souriau and Hudson, 2003). Expression levels of scFv or its derivatives in tobacco leaves vary from 0.1% (Fecker et al., 1996) to as high as 6.8% (Fiedler et al., 1997) of TSP. Moreover, by multiple sexual crossing of tobacco lines that is expressing individual non-overlapping scFvs, transgenic tobacco plants expressing more than one non-overlapping scFvs is being developed to provide optimal protective efficacy against multiple infections, such as against the Botulinum neurotoxin and anthrax simultaneously (Almquist et al., 2006). With the risk of such biological attacks, a source of inexpensive and rapidly produced yet effective antibodies is of the utmost importance.

Table 1
Examples of tobacco-made antibodies.

Disease targeted	Antibody expressed	Progress	Reference
Anthrax	Anti-PA mAb	Animal pre-clinical trial	(Hull et al., 2005)
Botulism	Anti-BoNT/A scFv	Animal pre-clinical trial	(Almquist et al., 2006)
Cancer (B-cell lymphoma)	Idiotype specifc	Human Phase I Clinical trial	(McCormick et al., 2008)
Cancer (breast and colon)	Anti-Lewis Y mAb	In vitro	(Brodzik et al., 2006)
Cancer (broad spectrum)	H10 mAb	In vitro	(Villani et al., 2009)
Cancer (colon)	CO17-1A mAb	Animal pre-clinical trial	(Ko et al., 2005)
Cancer (skin)	TheraCIM®	In vitro	(Rodriguez et al., 2005)
Hepatitis	B294, B303 mAb	In vitro	(Gleba et al., 2005)
HIV	2F5 mAb	In vitro	(Sack et al., 2007)
Rabies	R12 mAb	In vitro	(Girard et al., 2006)
S. mutans colonization	CaroRX™	Approved for sale	(Ma et al., 1998)
Salmonella	Anti-LPS scFv	In vitro	(Makvandi-Nejad et al., 2005)

Table 2
Examples of tobacco-made vaccines.

Disease targeted	Protein expressed	Progress	Reference
Allergy — dust mites	Der p 1	In vitro	(Lienard et al., 2007; Burtin et al., 2009)
	Der p 2	In vitro	(Lienard et al., 2007)
Antherax	Protective antigen	Animal pre-clinical trial	(Koya et al., 2005)
Cancer (cervical)	L1 major capsid protein	In vitro	(Lenzi et al., 2008)
Cholera	CTB	Animal pre-clinical trial	(Jani et al., 2004)
DPT	Multi-epitope vaccine	Animal pre-clinical trial	(Soria-Guerra et al., 2009)
Epstein–Barr virus	VCA antigen	In vitro	(Lee et al., 2006)
Foot and mouth disease	VP1	Animal pre-clinical trial	(Wu et al., 2003)
	VP21	Animal pre-clinical trial	(Huang et al., 2005)
Helicobacter pylori	HSP-A	Animal pre-clinical trial	(Brodzik et al., 2000)
	UreB	In vitro	(Gu et al., 2005)
Hepatitis B/C	HBsAg (Hep B)	Animal pre-clinical trial	(Thanavala et al., 1995)
	Core protein (Hep C)	In vitro	(Nianiou et al., 2008)
HIV	HIV p24 capsid protein	In vitro	(Zhang et al., 2002)
	HIV-Nef	In vitro	(Marusic et al., 2007)
Plague	F1-V	Animal pre-clinical trial	(Arlen et al., 2008; Del Prete et al., 2009)
SARS	SARS-CoV-S1 protein	Animal pre-clinical trial	(Pogrebnyak et al., 2005)
Tetanus	Tet-C	Animal pre-clinical trial	(Tregoning et al., 2005)
Type 1 diabetes	GAD65	Animal pre-clinical trial	(Ma et al., 2004)
	GAD67	Animal pre-clinical trial	(Ma et al., 1997)
	HSP-60	In vitro	(Tremblay et al., 2008)
	Insulin	Animal pre-clinical trial	(Li et al., 2006; Ruhlman et al., 2007)
Type 2 diabetes	GLP-1	In vitro	(Brandsma et al., 2009)

2.2. Vaccines

Vaccination is the administration of antigenic material (vaccine) to produce immunity to a disease. Vaccination is the most effective and cost-effective method of preventing infectious diseases. Traditionally, the vaccine administrated can either be live attenuated forms of pathogens such as bacteria or viruses, killed or inactivated forms of these pathogens. The development of subunit vaccines, based on the use of specific protein subunits of a bacterial pathogen or virus, will have less risk of adverse reactions than whole bacterial or virus vaccines. The use of recombinant DNA technology has simplified the production of subunit vaccines. Recombinant protein subunit vaccines are commonly produced in E. coli, yeast or mammalian cells cultures, but are limited by scale, product quality and production cost. Tobacco has emerged as a promising alternative expression system for production of recombinant subunit vaccines. To date, a large number of vaccine proteins have been produced in tobacco plants, ranging from vaccine candidates against foot and mouth disease, cholera, hepatitis B virus, severe acute respiratory syndrome (SARS) virus, human immunodeficiency virus (HIV) to vaccine candidates against various cancers (see Table 2). Many of tobacco-derived vaccines have already proven to be effective in animal testing. For example, Pogrebnyak et al. (2005) produced the SARS-CoV S1 protein in tobacco to prevent the spread of this highly contagious disease. They reported that mice primed with the tobacco made S1 protein developed an immune response by producing SARS-CoV-S1 specific IgG antibodies. Another example is from Golovkin et al. (2007) who report the production of a functional vaccinia virus B5 protein in tobacco as a potential vaccine. Mice immunized with tobacco-made B5 protein showed a strong anti-B5 immune response that was sufficient to protect the mice from lethal doses of small pox. Tobacco has also been used to produce vaccines for various veterinary applications. DOW AgroSciences received approval for the veterinary use of its Newcastle Disease vaccine for poultry, the first tobacco cell-derived vaccine approved by the United States Department of Agriculture (Vermij and Waltz, 2006).

The increased focus on tobacco products in recent years has led to their clinical evaluation. In a recent phase I clinical trial carried out, patients suffering from non-Hodgkin's lymphoma were given tobacco-derived personalized (patient-specific) idiotype single-chain antibodies (ScFv) as a vaccine (McCormick et al., 2008). These idiotypic single-chain ScFv antibodies were derived from each patient's tumor, hence allowing the immunization of patients with their own individual therapeutic antigen. The transient tobacco-viral expression system was used for rapid production of the needed amount of idiotype vaccine. The human trial results demonstrate that tobacco-derived idiotype vaccine is safe to administer, and stimulate idiotype-specific antibody and cellular immune responses.

2.3. Cytokines

Cytokines are small proteins or glycoproteins that are produced by a variety of cell types. They are strong immunoregulators that modulate the intensity and duration of the immune response by stimulating or inhibiting the activation, proliferation, and/or differentiation of various cells and by regulating their secretion of Abs or other cytokines (Parkin and Cohen, 2001). Cytokines exert their effects through binding to cytokine receptors expressed on the membrane of responsive target cells (Thomson and Lotze, 2003). Recombinant cytokines have been widely tested as therapeutic agents for the treatment of infectious and autoimmune diseases and cancers (Levy, 2006; Niedbala et al., 2008; Papaetis et al., 2008; Weigert et al., 2008). Although many recombinant cytokines are commercially available, they are too expensive to use therapeutically. Furthermore, there is a lack of glycosylation on most commercially available recombinant cytokines as most are E. coli-derived. Glycosylation is important for protein folding, stability and function. In recent years, transgenic tobacco has been exploited as a new source of inexpensive

recombinant cytokines. The functional expression of Human GM-CSF, human and murine IL-4, human, murine and viral IL-10, human IL-12, human IL-18 in tobacco plants or tobacco cell cultures have been reported (Table 3).

Human IL-13 is an example of a pleiotropic regulatory cytokine with the potential for treating several human diseases including Type-1 diabetes mellitus (T1DM). Thus far, conventional expression systems for recombinant human IL-13 production have proven difficult and are limited by efficiency. For example, low accumulation levels were achieved when mutant forms of hIL-13 bearing amino acid substitutions were expressed in *E. coli* (Thompson and Debinski, 1999). The accumulation level of hIL-13 in *E. coli* could be improved by co-expressing hIL-13 as a fusion with maltose binding protein containing an engineered tobacco etch virus recognition site at its C-terminus but TEV cleavage proved inefficient and required further processing to purify (Eisenmesser et al., 2000). Moreover, because the *E. coli*-derived unglycosylated rhIL-13 is misfolded and packs into inclusion bodies, additional solubilization and renaturation steps are required before a biologically active rhIL-13 can be produced (Thompson and Debinski, 1999). All of these make the use of *E. coli* for large-scale production of rhIL-13 at low cost unrealistic. The production of rhIL-13 in murine NS-O cells is also inefficient, as NS-O cell-derived rhIL-13 exists in both monomeric and trimeric forms (Cannon-Carlson et al., 1998). As the latter form has no biological activity, it must be separated from the biologically active monomeric form of rhIL-13 through a multi-step process, lowering production efficiency and raising costs. We have therefore tested low-nicotine low-alkaloid tobacco (cultivar '81V9') as a new expression host for recombinant human IL-13 production (Wang et al., 2008). We demonstrated that human IL-13 protein was efficiently accumulated in transgenic tobacco plants and present in multiple molecular forms, with an expression level as high as 0.15% of TSP in leaves. The multiple forms of tobacco-derived recombinant hIL-13 (rhIL-13) are due to differential N-linked glycosylation as revealed by enzymatic/chemical deglycosylation and not due to disulfide-linked oligomerization. In vitro trypsin digestion indicated that plant rhIL-13 is more resistant to proteolysis than unglycosylated control rhIL-13. The stability of plant rhIL-13 to digestion was further supported with simulated gastric and intestinal fluid digestion. In vitro bioassays using a factor-dependent human erythroleukemic cell line (TF-1 cells), showed that plant rhIL-13 retained the biological functions of the authentic hIL-13 protein. These results suggest that transgenic plants are superior over conventional cell-based expression systems for the production of rhIL-13.

3. Oral tolerance induction using tobacco expressing autoantigens

Oral administration of protein antigens can result in diminished peripheral immune responses to a subsequent systemic challenge with the same antigen in a process known as oral tolerance (Weiner, 1997). It is now well established that oral tolerance is a important natural physiological property of the immune system, whereby the host can avoid dangerous reactions such as DTH (delayed-type hypersensitivity) to "non-dangerous" antigens and other substances encountered in our daily diets (Mowat, 1987). Oral tolerance has been viewed as a potential therapeutic strategy for preventing and treating autoimmune diseases such as T1DM when specific disease-inducing self antigens (autoantigens) such as glutamic acid decarboxylase (GAD) are identified. With greater patient acceptance of oral rather than systemic therapies (e.g., by injection) and antigen specific effects without toxic effects of general immunosuppression, oral tolerance remains an attractive strategy that merits further clinical testing. Oral tolerance has been shown to suppress several autoimmune diseases in animal models, including experimental allergic encephalomyelitis (EAE), uveitis, thyroiditis, myasthenia, arthritis and T1DM in the non-obese diabetic (NOD) mouse, as well as other inflammatory conditions such as allergy and transplantation (Faria and Weiner, 2005). Oral tolerance induction has been further tested in human autoimmune diseases, including multiple sclerosis (MS), rheumatoid arthritis (RA), uveitis and T1DM. The results of human trials suggest that there is no systemic toxicity or exacerbation of disease (Faria and Weiner, 2005). As induction of oral tolerance requires repeated administration of large doses of antigen, however, clinical application of oral tolerance strategies may be restricted by the potentially huge cost of producing autoantigens. A new expression platform that provides large amounts of functional protein at extremely low cost is highly desired to make oral tolerance a more efficient and affordable therapeutic regimen. Transgenic plant expression systems have the potential to produce not only large amounts of protein economically, but also to be used as a vehicle for direct oral delivery of minimally processed peptides or proteins to induce oral tolerance.

Our group has been exploring transgenic tobacco plant as a primary vehicle for expression and direct oral delivery of T1DM-associated autoantigens such as GAD intended for inducing oral tolerance to prevent autoimmune diabetes. There are two isomers of GAD, GAD65 and 67, in rodents and humans, both of which are implicated in T1DM (Masteller and Bluestone, 2002). Rat and human islets express GAD65 predominantly, whereas mouse islets majorly express GAD67 (Elliott et al., 1994). Elliott et al. (1994) showed that systemic injection of GAD67 to young NOD mice specifically prevented the onset of diabetes. To provide initial proof of concept for the use of transgenic plants to induce oral immune tolerance, we therefore produced transgenic tobacco plants expressing mouse GAD67 to facilitate the design of animal experiments for testing plant-derived GAD (Ma et al., 1997). We showed for the first time that feeding GAD67 tobacco plants to young pre-diabetic female NOD prevented the development of diabetes.

As adjuvants facilitate tolerance induction and reduce doses of co-administered autoantigens, we were interested in finding effective mucosal adjuvants suitable for long-term administration for oral tolerance induction using transgenic plant-based systems. As one of the proposed mechanisms of oral tolerance is associated with an immune deviation, causing a shift in immune response from pro-inflammatory Th1 to anti-inflammatory Th2 response, Th2 cytokines such as IL-4 have been examined for possible enhancement of oral tolerance due to their known roles in differentiating precursor Th0 cells to Th2 lineages (Mosmann and Coffman, 1989). To test whether Th2 cytokine could be used to enhance oral immune tolerance to co-administered β-cell autoantigen, we generated transgenic tobacco plants expressing mouse IL-4 (mIL-4) and human GAD65 (hGAD65) (Ma et al., 2004). Tobacco-derived mIL-4 was found to retain its biological activity. Importantly, while feeding NOD mice with mIL-4 or hGAD65 tobacco alone had no effect on diabetes protection, combined feeding of mIL-4 and hGAD65 tobacco plants protected NOD mice from developing diabetes. Recently, Ruhlman et al. (2007) reported the production of chloroplast-transformed lettuce and tobacco plants that express an adjuvanted antigen consisting of cholera toxin B subunit (CTB) and proinsulin, and demonstrated that oral feeding of

Table 3
Examples of tobacco-made cytokines.

Cytokine	Applications	Progress	Reference
GM-CSF	Neutropenia, aplastic anemia	In vitro	(Kim et al., 2004)
IL-4	Cancer/chronic inflammation/ autoimmune disease	In vitro	(Ma et al., 2004)
IL-10	Chronic inflammation	In vitro	(Menassa et al., 2007)
IL-12	Cancer/disease protection	In vitro	(Gutierrez-Ortega et al., 2004)
IL-13	Cancer/chronic inflammation/ autoimmune disease	In vitro	(Wang et al., 2008)
IL-18	Cancer/chronic inflammation/ autoimmune disease	In vitro	(Zhang et al., 2003)

transgenic plant tissues resulted in a reduction in the degree of insulitis in NOD mice.

4. Tobacco expression systems

One of the most advantageous aspects of recombinant protein production in tobacco is the variety of expression strategies available, each with its own strengths. Stable nuclear transformation is able to meet long-term demands for authentic glycoproteins such as antibodies. Alternatively, chloroplast-based expression can provide large quantities of proteins that require minimal post-translational processing. When a product is required for rapid turnaround, as in the aforementioned production of an idiotype anti-cancer vaccine, transient expression would be expected to provide useful amounts of a desired protein in a very short period of time. Tobacco offers the flexibility to meet many different demands at once and has the potential to excel at each of them.

4.1. Nuclear expression

The tobacco nuclear genome is readily transformed via agrobacterium infection or biolistic delivery. Stable integration into the nuclear genome allows for continual recombinant protein production with little to no external input, simultaneously simplifying production and reducing costs. Nuclear expressed proteins undergo typical eukaryotic post-translational modifications and can be stored in a variety of organelles or secreted, depending on the fused signalling peptides. A major concern of nuclear based expression, however, is the relatively low-level accumulation of recombinant proteins. There exist a number of options that can address this issue, among them the use of strong constitutive/tissue specific promoters, 5′ enhancer sequences to increase translational efficiency, 3′ untranslated region modifications to increase transcript stability or optimizing the coding sequence of the transgene to use typical tobacco codons (Streatfield, 2007). Another common strategy is the use of a sub-cellular localization signal, allowing for the sorting of recombinant protein to specific sub-cellular locations in order to limit proteolytic degradation and hence increase accumulation (Benchabane et al., 2008). The most commonly targeted organelle for increased accumulation is the endoplasmic reticulum (ER) via the attachment of a C-terminal (K/H)DEL signal (Denecke et al., 1992). Following secretion to the golgi apparatus, proteins are returned to the ER where the low levels of proteolytic activity and a furtherance of complex molecular folding can result in higher levels of accumulation for many proteins. More recently, researchers have been exploring the localization of recombinant proteins into protein bodies. Protein bodies are typically formed by storage proteins found in seeds and stable, inert compartments produced as buds from the endoplasmic reticulum that are highly resistant to proteolytic degredation (Torrent et al., 2009a,b). By adding a protein body forming signal peptide, referred to as a zein, a desired recombinant protein can be expressed and retained in these protein bodies, allowing for potentially higher levels of accumulation, with one study showing between 10- to 100-fold increased accumulation of the fused recombinant protein (Torrent et al., 2009a,b).

Another method to increase recombinant protein accumulation is to add fusion partners that can increase protein stability/proteolysis resistance. The elastin-like polypeptide (ELP) is one such example. ELP is composed of pentapeptide repeats (VPGXG) that upon the addition of heat and/or salt undergoes a reversible change of conformation that results in the formation of an immiscible layer in water that allows for simplified downstream purification and have been reported to increase accumulation levels of fused proteins in tobacco (Patel et al., 2007; Floss et al., 2008). Indeed, when used in a transient expression system, fusion with ELP led to nearly twice the level of GFP accumulation in the ER due to the ability of ELP to induce

the formation of protein bodies (Conely et al., 2009). Fusion partners that increase stability and enhance immunogenicity are effective in tobacco when a small, unstable antigenic peptide is desired, the most common being the non-toxic subunit of cholera toxin B (CTB) (Arakawa et al., 1998). CTB enhances the stability of fused antigens through the formation of a proteolytic-resistant pentameric structure. It also serves as an effective adjuvant that can enhance the immunogenicity of fused protein during oral delivery, as its pentameric structure efficiently binds to GM-1 gangliosides that are involved in antigen display (Holmgren et al., 1993). This has allowed for the production of unstable peptide antigens that were previously unattainable in tobacco due to their small size including the B-chain of insulin and the p277 fragment of HSP60 (Daniell et al., 2001a,b; Li et al., 2006; Tremblay et al., 2008).

Another concern of tobacco (plant in general) bioreactors is in the generation of therapeutic glycoproteins. While tobacco is able to create complex glycoproteins, they contain plant-specific glycans. The main complication is the addition of both $\alpha 1,3$-fucose and $\beta 1,2$-xylose residues, both of which are known IgE binding carbohydrate factors in plant allergens. In some instances this is adventitious, especially where the antibodies are intended to function as antigens. In some cases it is detrimental, however, and results in either a glycoprotein that is ineffectual or harmful to the host as seen in the case of a monoclonal murine antibody that lacked immunogenicity due to its non-authentic glycosylation (Chargelegue et al., 2000). A number of approaches have been developed to produce more authentic glycoproteins. For example, several groups have achieved successful knock-down in plant transferase activity via RNA-interference (RNAi), resulting in a shift in protein glycosylation away from plant-type glycans to the production of simple glycans that lack IgE activity (Sourrouille et al., 2008; Strasser et al., 2008). Another approach involves the expression of a human $\beta 1,4$-galactosyltransferase I that was able to generate a monoclonal antibody that was nearly identical to the standard made in hamster CHO cells (Bakker et al., 2006). By fusing the human galactosyltransferase with a plant N-terminal domain, they were able to sequester the transferase to the early golgi apparatus, resulting in human N-glycans being added before tobacco N-glycans are typically added. For tobacco cell culture, there is another method to influence glycoprotein composition by altering the make-up of the growth media. By using a media rich in glucosamine, researchers were able to increase the ratio of simple to complex glycans from 6% to 66% in tobacco NT-1 cells (Becerra-Arteaga and Shuler, 2007).

4.2. Chloroplastic expression

Tobacco chloroplast offers an alternative stable expression system to nuclear transformation that has been shown to accumulate significant levels of recombinant protein. For example, Ruhlman et al. (2007) reported that a chloroplastic expressed CTB-insulin fusion had a 160-fold increase in accumulation over a similar construct that was nuclear expressed (Ruhlman et al., 2007). Chloroplast transformation has been further refined and developed into a highly efficient recombinant protein expression system since the first biolistic delivery of recombinant DNA into chloroplasts in the late 1980s (Maliga, 2004). Incorporation into the chloroplast genome occurs through homologous recombination and is typically targeted to the inverted repeat region, reducing the extensive screening required for genomic transformation due to positional effects. While the level of accumulation appears to vary depending on the expression cassette used and the type of protein being expressed, it is not uncommon to see levels of expression range from 5 to 20% TSP, an astounding amount given that current nuclear transformation techniques have typical expression range of 0.001 to ~1% TSP (Daniell et al., 2001a,b; Grevich and Daniell, 2005). More recently, Bally et al. (2009) performed a detailed analysis of recombinant protein

production in chloroplasts in order to determine why plants remain metabolically healthy despite the alteration in resources caused by massive protein accumulation. They found that Rubisco, the major protein in plants, acts as a protein sink and exists naturally in quantities greater than metabolically required. Recombinant protein production replaces Rubisco as the protein sink, leading to lower Rubisco levels with rising recombinant protein accumulation, allowing for a measure of natural capacity for recombinant protein production in plants without affecting growth and development. A second advantage of expressing and accumulating proteins in tobacco chloroplasts is in transgene containment. In plants, pollen donates its DNA to the egg cell, similar to embryo fertilization in humans. As with humans, organelles such as chloroplasts and mitochondria of the offspring are solely maternal in origin. Therefore, genes expressed in chloroplasts, and by extension other plastids, are highly unlikely to be transmitted through pollen. This has been supported by recent work that demonstrated that following fertilization with pollen containing chloroplast expressed resistance marker, only 6 seedlings from a population of over 2 million progeny showed paternal transmission (Ruf et al., 2007). Therefore, when combined with male sterile lines and traditional leaf specific expression and harvest, chloroplast expression virtually guarantees transgene containment in tobacco. A limitation of chloroplast recombinant protein production is that like bacteria they are unable to perform glycosylation, a necessity for many pharmaceutical glycoproteins including monoclonal antibodies. It is important to note, however, that the basic building blocks, the glycans themselves, are present within the chloroplast (Fettke et al., 2004). Thus it may be possible to generate glycoproteins in chloroplasts with the addition of multiple steps of the glycan addition/modification pathway, although this has yet to be described in literature.

4.3. Transient expression

Transient expression in tobacco allows for the rapid production of a required protein. In as little as 5 days post infection, significant quantities of recombinant protein can be generated and harvested, allowing for rapid turnaround and analysis that is unachievable via stable genomic integration. Many of the advancements in sub-cellular localization and fusions described above for nuclear transformation also hold true for transient expression. Most of the current transient expression systems used in tobacco for pharmaceutical production use viral coding sequences delivered by *Agrobacterium tumicfaciens* as it provides high level expression with minimal input. In order to maximize the efficiency of transient expression, various strategies are emerging that hold the potential to drastically increase yield. One promising technology is "Magnifection" that is designed to minimize the amount of wasted energy used if creating viral particles by using agrobacterium to deliver a deconstructed virus directly to the cells (Gleba et al., 2005). This method involves the delivery of individual components of the viral expression platform by mixing different agrobacterium lines harbouring fractions of the viral machinery, with recombination occurring intracellularly once infection occurs. By further altering the codon usage of the virus and the inclusion of typical eukaryotic introns, they were able to drastically increase the efficiency of delivery, resulting in a reduction in the amount of required infectious agrobacterium (i.e., 1 l of an overnight culture could be used to infect nearly 1000 kg of tobacco leaf tissue, yielding up to 4 kg of recombinant protein at 40% TSP; Marillonnet et al., 2005). This exemplifies the measure of approaches that are currently being explored to maximize protein yield while minimizing input costs, resulting in an estimated $1/kg of raw protein or $50/kg purified. An additional option is to insert the viral machinery required to inhibit tobacco silencing via stable nuclear transformation first, further minimizing the delivery requirements of the deconstructed viral vectors themselves (Azhakanandam et al., 2007). There are two major

drawbacks for transient expression, however. First are the technical requirements for induction, as it requires mechanical delivery of the agrobacterium to the plant. A second drawback is due to the high risk of unintended spread of the infection to wild species, which necessitates this work must be confined to laboratories and greenhouse conditions, although this is less of a concern as it eliminates the risks of cross-fertilization normally associated with open-field grown transgenic plants in general.

5. Current and future regulation

Plants are poised to become a new route for a major source of inexpensive pharmaceuticals. Plant-derived pharmaceuticals will need to meet the same safety and efficacy standards as those products obtained from non-plant sources. Many countries have, or are in the process of creating, established guidelines and regulatory policies on plant-derived therapeutics. In the U.S., plant-made pharmaceutical research, development, testing and production is reviewed and regulated by the U.S. Department of Agriculture (USDA) and the Food and Drug administration (FDA). USDA and FDA regulations regarding development, testing, production, transportation and commercialization of plant-made pharmaceuticals are expected to adapt as the technology advances. Currently, a USDA field permit is required to plant crops that produce plant-made pharmaceuticals, with permits granted on a case-by-case basis. In Canada, experimental field testing of biopharmaceutical crops is also subjected to government regulation, requiring a pre-approved permit. It is anticipated that government approval and regulation of plant-made pharmaceuticals may be as strict as traditional ethical pharmaceuticals.

6. Conclusion

Plants are proving to be effective and efficient bioreactors for the production of pharmaceutically valuable recombinant proteins. There are a variety of plant species that are being explored to serve as green bioreactors, each with its own advantages and disadvantages. Tobacco offers a unique blend of biomass accumulation, ease of manipulation, protection against food-chain contamination and bio-containment that is not readily found in other plant species. With further technological development and an expansion in the range of products, the future for this oft-maligned plant has the chance to become a positive force to improve the quality of life for all.

Acknowledgements

References

Almquist KC, McLean MD, Niu YQ, Byrne G, Olea-Popelka FC, Murrant C, et al. Expression of an anti-botulinum toxin A neutralizing single-chain Fv recombinant antibody in transgenic tobacco. Vaccine 2006;24:2079–86.

Arakawa T, Yu J, Chong DKX, Hough J, Engen PC, Langridge WHR. A plant-based cholera toxin B subunit–insulin fusion protein protects against the development of autoimmune diabetes. Nat Biotechnol 1998;16:934–8.

Arlen PA, Singleton M, Adamovicz JJ, Ding Y, Davoodi-Semiromi A, Daniell H. Effective plague vaccination via oral delivery of plant cells expressing F1-V antigens in chloroplasts. Infect Immun 2008;76:3640–50.

Azhakanandam K, Weissinger SM, Nicholson JS, Qu RD, Weissinger AK. Amplicon-plus Targeting Technology (APTT) for rapid production of a highly unstable vaccine protein in tobacco plants. Plant Mol Biol 2007;63:393–404.

Bakker H, Rouwendal GJA, Karnoup AS, Florack DEA, Stoopen GM, Helsper J, et al. An antibody produced in tobacco expressing a hybrid beta-1, 4-galactosyltransferase is essentially devoid of plant carbohydrate epitopes. Proc Natl Acad Sci U S A 2006;103:7577–82.

Bally J, Nadai M, Vitel M, Rolland A, Dumain R, Dubald M. Plant physiological adaptations to the massive foreign protein synthesis occurring in recombinant chloroplasts. Plant Physiol 2009;150:1474–81.

Becerra-Arteaga A, Shuler ML. Influence of culture medium supplementation of tobacco NT1 cell suspension cultures on the N-glycosylation of human secreted alkaline phosphatase. Biotechnol Bioeng 2007;97:1585–93.

Benchabane M, Goulet C, Rivard D, Faye L, Gomord V, Michaud D. Preventing unintended proteolysis in plant protein biofactories. Plant Biotechnol J 2008;6:633–48.

Brandsma M, Wang X, Diao H, Kohalmi S, Jevnikar A, Ma S. A proficient approach to the production of therapeutic glucagon-like peptide-1 (GLP-1) in transgenic plants. Open Biotechnol J 2009;3:57–66.

Brodzik R, Koprowski H, Yusibov V, Sirko A. Production of urease from Helicobacter pylori in transgenic tobacco plants. Cell Mol Biol Lett 2000;5:357–66.

Brodzik R, Glogowska M, Bandurska K, Okulicz M, Deka D, Ko K, et al. Plant-derived anti-Lewis Y mAb exhibits biological activities for efficient immunotherapy against human cancer cells. Proc Natl Acad Sci U S A 2006;103:8804–9.

Burtin D, Chabrew H, Olagnier B, Didierlaurent A, Couret MN, Comeau D, et al. Production of native and modified recombinant Der p 1 molecules in tobacco plants. Clin Exp Allergy 2009;39:760–70.

Cannon-Carlson S, Varnerin J, Tsarbopoulos A, Jenh CH, Cox MA, Chou CC, et al. Expression, purification, and characterization of recombinant human interleukin-13 from NS-0 cells. Protein Expr Purif 1998;12:239–48.

Chargelegue D, Vine ND, van Dolleweerd CJ, Drake PMW, Ma JKC. A murine monoclonal antibody produced in transgenic plants with plant-specific glycans is not immunogenic in mice. Transgenic Res 2000;9:187–94.

Conely AJ, Joensuu JJ, Menass R, Brandle JE. Induction of protein body formation in plant leaves by elastin-like polypeptide fusions. BMC Biology 2009;7:48.

Daniell H, Lee SB, Panchal T, Wiebe PO. Expression of the native cholera toxin B subunit gene and assembly as functional oligomers in transgenic tobacco chloroplasts. J Mol Biol 2001a;311:1001–9.

Daniell H, Streatfield SJ, Wycoff K. Medical molecular farming: production of antibodies, biopharmaceuticals and edible vaccines in plants. Trends Plant Sci 2001b;6:219–26.

Del Prete G, Santi L, Andrianaivoarimanana V, Amedei A, Domarle O, D'Elios MM, et al. Plant-derived recombinant F1, V, and F1-V fusion antigens of Yersinia pestis activate human cells of the innate and adaptive immune system. Int J Immunopathol Pharmacol 2009;22:133–43.

Denecke J, Derycke R, Botterman J. Plant and mammalian sorting signals for protein retention in the endoplasmic-reticulum contain a conserved epitope. Embo J 1992;11:2345–55.

Eisenmesser EZ, Kapust RB, Nawrocki JP, Mazzulla MJ, Pannell LK, Waugh DS, et al. Expression, purification, refolding, and characterization of recombinant human interleukin-13: utilization of intracellular processing. Protein Expr Purif 2000;20:186–95.

Elliott JF, Qin HY, Bhatti S, Smith DK, Singh RK, Dillon T, et al. Immunization with the larger isoform of mouse glutamic-acid decarboxylase (GAD(67)) prevents autoimmune diabetes in NOD mice. Diabetes 1994;43:1494–9.

Faria AMC, Weiner HL. Oral tolerance. Immunol Rev 2005;206:232–59.

Fecker LF, Kaufmann A, Commandeur U, Commandeur J, Koenig R, Burgermeister W. Expression of single-chain antibody fragments (scFv) specific for beet necrotic yellow vein virus coat protein or 25 kDa protein in Escherichia coli and Nicotiana benthamiana. Plant Mol Biol 1996;32:979–86.

Fettke J, Eckermann N, Poeste S, Pauly M, Steup M. The glycan substrate of the cytosolic (Pho 2) phosphorylase isozyme from Pisum sativum L.: identification, linkage analysis and subcellular localization. Plant J 2004;39:933–46.

Fiedler U, Phillips J, Artsaenko O, Conrad U. Optimization of scFv antibody production in transgenic plants. Immunotechnology 1997;3:205–16.

Floss DM, Sack M, Stadlmann J, Rademacher T, Scheller J, Stoger E, et al. Biochemical and functional characterization of anti-HIV antibody-ELP fusion proteins from transgenic plants. Plant Biotechnol J 2008;6:379–91.

Girard LS, Fabis MJ, Bastin M, Courtois D, Petiard V, Koprowski H. Expression of a human anti-rabies virus monoclonal antibody in tobacco cell culture. Biochem Biophys Res Commun 2006;345:602–7.

Gleba Y, Klimyuk V, Marillonnet S. Magnifection — a new platform for expressing recombinant vaccines in plants. Vaccine 2005;23:2042–8.

Golovkin M, Spitsin S, Andrianov V, Smirnov Y, Xiao YH, Pogrebnyak N, et al. Smallpox subunit vaccine produced in planta confers protection in mice. Proc Natl Acad Sci U S A 2007;104:6864–9.

Grevich JJ, Daniell H. Chloroplast genetic engineering: recent advances and future perspectives. Crit Rev Plant Sci 2005;24:83-107.

Gu Q, Han N, Liu J, Zhu M. Cloning of Helicobacter pylori urease subunit B gene and its expression in tobacco (Nicotiana tabacum L.). Plant Cell Rep 2005;24:532–9.

Gutierrez-Ortega A, Avila-Moreno F, Saucedo-Arias LJ, Sanchez-Torres C, Gomez-Lim MA. Expression of a single-chain human interleukin-12 gene in transgenic tobacco plants and functional studies. Biotechnol Bioeng 2004;85:734–40.

Hiatt A, Cafferkey R, Bowdish K. Production of antibodies in transgenic plants. Nature 1989;342:76–8.

Holmgren J, Lycke N, Czerkinsky C. Cholera-toxin and cholera-B subunit as oral mucosal adjuvant and antigen vector systems. Vaccine 1993;11:1179–84.

Horn ME, Woodard SL, Howard JA. Plant molecular farming: systems and products. Plant Cell Rep 2004;22:711–20.

Huang Y, Liang W, Wang Y, Zhou Z, Pan A, Yang X, et al. Immunogenicity of the epitope of the foot-and-mouth disease virus fused with a hepatitis B core protein as expressed in transgenic tobacco. Viral Immunol 2005;18:668–77.

Hull AK, Criscuolo CJ, Mett V, Groen H, Steeman W, Westra H, et al. Human-derived, plant-produced monoclonal antibody for the treatment of anthrax. Vaccine 2005;23:2082–6.

Jani D, Singh NK, Bhattacharya S, Meena LS, Singh Y, Upadhyay SN, et al. Studies on the immunogenic potential of plant-expressed cholera toxin B subunit. Plant Cell Rep 2004;22:471–7.

Johansen FE, Pekna M, Norderhaug IN, Haneberg B, Hietala MA, Krajci P, et al. Absence of epithelial immunoglobulin A transport, with increased mucosal leakiness, in polymeric immunoglobulin receptor/secretory component-deficient mice. J Exp Med 1999;190:915–22.

Kim YS, Kwon TH, Sik YM. Direct transfer and expression of human GM-CSF in tobacco suspension cell using Agrobacterium-mediated transfer system. Plant Cell Tissue Organ Cult 2004;78:133–8.

Ko K, Steplewski Z, Glogowska M, Koprowski H. Inhibition of tumor growth by plant-derived mAb. Proc Natl Acad Sci U S A 2005;102:7026–30.

Koya V, Moayeri M, Leppla SH, Daniell H. Plant-based vaccine: mice immunized with chloroplast-derived anthrax protective antigen survive anthrax lethal toxin challenge. Infect Immun 2005;73:8266–74.

Lee MYT, Zhou YX, Lung RWM, Chye ML, Yip WK, Zee SY, et al. Expression of viral capsid protein antigen against Epstein–Barr virus in plastids of Nicotiana tabacum cv. SR1. Biotechnol Bioeng 2006;94:1129–37.

Lehner T, Caldwell J, Smith R. Local passive immunization by monoclonal antibodies against streptococcal antigen I/II in the prevention of dental caries. Infect Immun 1985;50:796–9.

Lenzi P, Scotti N, Alagna F, Tornesello ML, Pompa A, Vitale A, et al. Translational fusion of chloroplast-expressed human papillomavirus type 16L1 capsid protein enhances antigen accumulation in transplastomic tobacco. Transgenic Res 2008;17:1091–102.

Levy Y. Cytokine therapies in HIV infection. M S-Med Sci 2006;22:751–4.

Li D, O'Leary J, Huang Y, Huner NP, Jevnikar AM, Ma S. Expression of cholera toxin B subunit and the B chain of human insulin as a fusion protein in transgenic tobacco plants. Plant Cell Rep 2006;25:417–24.

Lienard D, Dinh OT, van Oort E, Van Overtvelt L, Bonneau C, Wambre E, et al. Suspension-cultured BY-2 tobacco cells produce and mature immunologically active house dust mite allergens. Plant Biotechnol J 2007;5:93-108.

Ma JK, Hunjan M, Smith R, Lehner T. Specificity of monoclonal antibodies in local passive immunization against Streptococcus mutans. Clin Exp Immunol 1989;77:331–7.

Ma JKC, Hiatt A, Hein M, Vine ND, Wang F, Stabila P, et al. Generation and assembly of secretory antibodies in plants. Science 1995;268:716–9.

Ma S, Zhao DL, Yin ZQ, Mukherjee R, Singh B, Qin HY, et al. Transgenic plants expressing autoantigens fed to mice to induce oral immune tolerance. Nat Med 1997;3:793–6.

Ma JKC, Hikmat BY, Wycoff K, Vine ND, Chargelegue D, Yu L, et al. Characterization of a recombinant plant monoclonal secretory antibody and preventive immunotherapy in humans. Nat Med 1998;4:601–6.

Ma JKC, Drake PMW, Christou P. The production of recombinant pharmaceutical proteins in plants. Nat Rev Genet 2003;4:794–805.

Ma S, Huang Y, Yin Z, Menassa R, Brandle JE, Jevnikar AM. Induction of oral tolerance to prevent diabetes with transgenic plants requires glutamic acid decarboxylase (GAD) and IL-4. Proc Natl Acad Sci U S A 2004;101:5680–5.

Makvandi-Nejad S, McLean MD, Hirama T, Almquist KC, MacKenzie CR, Hall JC. Transgenic tobacco plants expressing a dimeric single-chain variable fragment (scFv) antibody against Salmonella enterica serotype paratyphi B. Transgenic Res 2005;14:785–92.

Maliga P. Plastid transformation in higher plants. Annu Rev Plant Biol 2004;55:289–313.

Marillonnet S, Thoeringer C, Kandzia R, Klimyuk V, Gleba Y. Systemic Agrobacterium tumefaciens-mediated transfection of viral replicons for efficient transient expression in plants. Nat Biotechnol 2005;23:718–23.

Marusic C, Nuttall J, Buriani G, Lico C, Lombardi R, Baschieri S, et al. Expression, intracellular targeting and purification of HIV Nef variants in tobacco cells. BMC Biotechnol 2007;7:12.

Masteller EL, Bluestone JA. Immunotherapy of insulin-dependent diabetes mellitus. Curr Opin Immunol 2002;14:652–9.

McCormick AA, Reddy S, Reinl SJ, Cameron TI, Czerwinkski DK, Vojdani F, et al. Plant-produced idiotype vaccines for the treatment of non-Hodgkin's lymphoma: safety and immunogenicity in a phase I clinical study. Proc Natl Acad Sci U S A 2008;105:10131–6.

Menassa R, Nguyen V, Jevnikar A, Brandle J. A self-contained system for the field production of plant recombinant interleukin-10. Mol Breeding 2001;8:177–85.

Menassa R, Du C, Yin ZQ, Ma S, Poussier P, Brandle J, et al. Therapeutic effectiveness of orally administered transgenic low-alkaloid tobacco expressing human interleukin-10 in a mouse model of colitis. Plant Biotechnol J 2007;5:50–9.

Mosmann TR, Coffman RL. TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. Annu Rev Immunol 1989;7:145–73.

Mowat AM. The regulation of immune-responses to dietary-protein antigens. Immunol Today 1987;8:93–8.

Nianiou I, Kalantidis K, Madesis P, Georgopoulou U, Mavromara P, Tsaftaris A. Expression of an HCV core antigen coding gene in tobacco (N. tabacum L.). Prep Biochem Biotechnol 2008;38:411–21.

Niedbala W, Cai B, Wei X, Patakas A, Leung BP, McInnes IB, et al. Interleukin 27 attenuates collagen-induced arthritis. Ann Rheum Dis 2008;67:1474–9.

Papaetis GS, Karapanagiotou LM, Pandha H, Syrigos KN. Targeted therapy for advanced renal cell cancer: cytokines and beyond. Curr Pharm Des 2008;14:2229–51.

Parkin J, Cohen B. An overview of the immune system. Lancet 2001;357:1777–89.

Patel J, Zhu H, Menassa R, Gyenis L, Richman A, Brandle J. Elastin-like polypeptide fusions enhance the accumulation of recombinant proteins in tobacco leaves. Transgenic Res 2007;16:239–49.

Pogrebnyak N, Golovkin M, Andrianov V, Spitsin S, Smirnov Y, Egolf R, et al. Severe acute respiratory syndrome (SARS) S protein production in plants: development of recombinant vaccine. Proc Natl Acad Sci U S A 2005;102:9062–7.

Rodriguez M, Ramirez NI, Ayala M, Freyre F, Perez L, Triguero A, et al. Transient expression in tobacco leaves of an aglycosylated recombinant antibody against the epidermal growth factor receptor. Biotechnol Bioeng 2005;89:188–94.

Ruf S, Karcher D, Bock R. Determining the transgene containment level provided by chloroplast transformation. Proc Natl Acad Sci U S A 2007;104:6998–7002.

Ruhlman T, Ahangari R, Devine A, Samsam M, Daniell H. Expression of cholera toxin B-proinsulin fusion protein in lettuce and tobacco chloroplasts—oral administration protects against development of insulitis in non-obese diabetic mice. Plant Biotechnol J 2007;5:495–510.

Sack M, Paetz A, Kunert R, Bomble M, Hesse F, Stiegler G, et al. Functional analysis of the broadly neutralizing human anti-HIV-1 antibody 2F5 produced in transgenic BY-2 suspension cultures. Faseb J 2007;21:1655–64.

Soria-Guerra RE, Alpuche-Solis AG, Rosales-Mendoza S, Moreno-Fierros L, Bendik EM, Martinez-Gonzalez L, et al. Expression of a multi-epitope DPT fusion protein in transplastomic tobacco plants retains both antigenicity and immunogenicity of all three components of the functional oligomer. Planta 2009;229:1293–302.

Souriau C, Hudson PJ. Recombinant antibodies for cancer diagnosis and therapy. Expert Opin Biol Ther 2003;3:305–18.

Sourrouille C, Marquet-Blouin E, D'Aoust MA, Kiefer-Meyer MC, Seveno M, Pagny-Salehabadi S, et al. Down-regulated expression of plant-specific glycoepitopes in alfalfa. Plant Biotechnol J 2008;6:702–21.

Strasser R, Stadlmann J, Schahs M, Stiegler G, Quendler H, Mach L, et al. Generation of glyco-engineered *Nicotiana benthamiana* for the production of monoclonal antibodies with a homogeneous human-like N-glycan structure. Plant Biotechnol J 2008;6:392–402.

Streatfield SJ. Approaches to achieve high-level heterologous protein production in plants. Plant Biotechnol J 2007;5:2-15.

Thanavala Y, Yang YF, Lyons P, Mason HS, Arntzen C. Immunogenicity of transgenic plant-derived hepatitis-B surface-antigen. Proc Natl Acad Sci U S A 1995;92:3358–61.

Thompson JP, Debinski W. Mutants of interleukin 13 with altered reactivity toward interleukin 13 receptors. J Biol Chem 1999;274:29944–50.

Thomson AW, Lotze MT, editors. The cytokine handbook. Boston: Academic Press; 2003.

Torrent M, Llompart B, Lasserre-Ramassamy S, Llop-Tous I, Bastida M, Marzabal P, et al. Eukaryotic protein production in designed storage organelles. BMC Biol 2009a;7:14.

Torrent M, Llop-Tous I, Ludevid MD. Protein body induction: a new tool to produce and recover recombinant proteins in plants. Humana Press Inc: Methods in molecular biology. L. Faye and V. Gomord; 2009b. 193–208.

Tregoning JS, Clare S, Bowe F, Edwards L, Fairweather N, Qazi O, et al. Protection against tetanus toxin using a plant-based vaccine. Eur J Immunol 2005;35:1320–6.

Tremblay R, Wang X, Jevnikar AM, Ma S. Expression of a fusion protein consisting of cholera toxin B subunit and anti-diabetic peptide (p277) from human heat shock protein in transgenic tobacco plants. Transgenic Plant J 2008;2:186–91.

Vermij P, Waltz E. USDA approves the first plant-based vaccine. Nat Biotechnol 2006;24:233–4.

Villani ME, Morgun B, Brunetti P, Marusic C, Lombardi R, Pisoni I, et al. Plant pharming of a full-sized, tumour-targeting antibody using different expression strategies. Plant Biotechnol J 2009;7:59–72.

Wang DJ, Brandsma M, Yin ZQ, Wang AM, Jevnikar AM, Ma S. A novel platform for biologically active recombinant human interleukin-13 production. Plant Biotechnol J 2008;6:504–15.

Weigert C, Rocken M, Ghoreschi K. Interleukin 4 as a potential drug candidate for psoriasis. Expert Opin Drug Discov 2008;3:357–68.

Weiner HL. Oral tolerance for the treatment of autoimmune diseases. Annu Rev Med 1997;48:341–51.

Wu L, Jiang L, Zhou Z, Fan J, Zhang Q, Zhu H, et al. Expression of foot-and-mouth disease virus epitopes in tobacco by a tobacco mosaic virus-based vector. Vaccine 2003;21:4390–8.

Zhang GG, Rodrigues L, Rovinski B, White KA. Production of HIV-1 p24 protein in transgenic tobacco plants. Mol Biotechnol 2002;20:131–6.

Zhang B, Yang YH, Lin YM, Rao Q, Zheng GG, Wu KF. Expression and production of bioactive human interleukin-18 in transgenic tobacco plants. Biotechnol Lett 2003;25:1629–35.