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TITLE:
Recent developments in therapeutic protein expression technologies in plants

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.

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.

Plant system versus other heterologous protein expression systems ::: Background:
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 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 CO2 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 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 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.