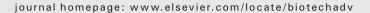Contents lists available at ScienceDirect

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Research review paper

Advances in plant molecular farming

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ABSTRACT

Plant molecular farming (PMF) is a new branch of plant biotechnology, where plants are engineered to produce recombinant pharmaceutical and industrial proteins in large quantities. As an emerging subdivision of the biopharmaceutical industry, PMF is still trying to gain comparable social acceptance as the already established production systems that produce these high valued proteins in microbial, yeast, or mammalian expression systems. This article reviews the various cost-effective technologies and strategies, which are being developed to improve yield and quality of the plant-derived pharmaceuticals, thereby making plant-based production system suitable alternatives to the existing systems. It also attempts to overview the different novel plant-derived pharmaceuticals and non-pharmaceutical protein products that are at various stages of clinical development or commercialization. It then discusses the biosafety and regulatory issues, which are crucial (if strictly adhered to) to eliminating potential health and environmental risks, which in turn is necessary to earning favorable public perception, thus ensuring the success of the industry.

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1. Introduction

Plant molecular farming (PMF) refers to the production of recombinant proteins (including pharmaceuticals and industrial proteins) and other secondary metabolites, in plants. This involves the growing, harvesting, transport, storage, and downstream processing of extraction and purification of the protein (Wilde et al., 2002). This technology hinges on the genetic transformability of plants, which was first demonstrated in the 1980s (Bevan et al., 1983). The first recombinant plant-derived pharmaceutical protein (the human growth hormone) and the first recombinant antibody (expressed in the progeny of the cross of two individual transgenic plants expressing single immunoglobulin gamma and kappa chains) were produced in transgenic plants in 1986 and 1989, respectively (Hiatt et al., 1989; Barta et al., 1986). However, it was not until 1997 that the first recombinant protein, avidin (an egg protein) was expressed in transgenic maize for commercial purpose (Hood et al., 1997). These exploits really demonstrated that plants could be turned into bio-factories for the large scale production of recombinant proteins. It has been proven over the years that plants have the ability to produce even more complex functional mammalian proteins with therapeutic activity, such as human serum proteins and growth regulators, antibodies, vaccines, hormones, cytokines, enzymes and antibodies (Liénard et al., 2007). This has been possible due to their ability to perform post-translational modifications that make these recombinant proteins fold properly and maintain their structural and functional integrity.

The fast-growing market for recombinant biopharmaceuticals, which accounted for about 10% of the pharmaceutical market in 2007 (Lowe and Jones, 2007), had been predicted to expand to US $ 100 billion in 2010 (Knäblein, 2005). With increasing demand for bio-pharmaceuticals, coupled with the high costs and inefficiency of the existing production systems (Knäblein, 2005), which include yeast, microbes, animals cells (Jones et al., 2003) and transgenic animals (Harvey et al., 2002), there now exists a shortage in manufacturing capacity, to such an extent that some patients have to wait for these products. Hence transgenic plants are gaining more attention as the new generation bio-reactors; with active research and development activity in PMF identified in over one hundred and twenty companies, universities, and research institutes, around the world (Basaran and Rodríguez-Cerezo, 2008). The weightier advantages of the plant-based system over the existing systems are the incentives for this. This review also discusses the different technologies and the limitations of the plant system with respect to the various approaches being pursued to overcome the challenges in other to increase its social acceptability. It also attempts to give an overview of the plant-derived proteins and discusses the biosafety and regulatory issues of these emerging technologies.

2. Plant transformation strategies

2.1. Stable nuclear transformation

Stable nuclear transformation involves the incorporation of a foreign gene or genes (exogenous) of interest into the nuclear genome of the plant, thereby altering its genetic makeup, and leading to the expression of the transgene after integration with the host genome, thereby conferring stably inheritable traits that were not present in the untransformed host plant. As the most common method, stable nuclear transformation has produced most of the recombinant proteins till date. The method has been used to accumulate protein in the dry seeds of cereals, which allows long-term storage of the seed at room temperature without degradation of the protein (Horn et al., 2004). Additionally, the system has high scale-up capacity, as crops such as the cereals are grown practically everywhere. However, a long production cycle of some crops and the potential to cross with native species or food crops are limiting public acceptance of this method (Commandeur et al., 2003).

2.2. Stable plastid transformation

Plastid transformation provides a valuable alternative to nuclear transformation because it combines numerous advantages that the nuclear transformation lacks, including the provision of a natural biocontainment of transgene flow by out-crossing (as plastids are inherited through maternal tissues in most species and the pollen does not contain chloroplasts, hence the transgene may not be transferable, thereby allaying public concerns (Cardi et al., 2010; Meyers et al., 2010). Transgenic plants with homoplastomic chloroplast transformation (in which every chloroplast carries the transgene) are selected after several generations of plant regeneration from bombarded leaf explants, on selection medium containing spectinomycin or in combination with streptomycin. Our group (Reddy et al., 2002; Sadhu and Reddy, 2003) had previously achieved accumulation of a bacterial- and a human therapeutic protein, up to 3–6% of total soluble proteins in tobacco chloroplasts. Recently, Oey et al. (2009) reported a whopping expression level (70% of the total soluble proteins) for a proteinaceous antibiotic, with the chloroplast system, which represents the highest recombinant protein accumulation achieved so far in plants. This enormous potentials notwithstanding, plastid transformation is still limited in its applications, in that even though it has been achieved in other plant species such as tomato, eggplant, lettuce, and soybeans (Bock, 2007; Singh and Verma, 2010), routine chloroplast transformation is only possible in tobacco, which is inedible and highly regulated, being rich in toxic alkaloids. It is also envisaged that protein stability will change over time even with refrigeration (Horn et al., 2004).

2.3. Plant cell-suspension cultures

Plant cell-suspension culture is an alternative plant-based production platform to mammalian cells for production of biopharmaceutical. The system guarantees sterile *in vitro* conditions, coupled with high-level containment (Franconi et al., 2010) (ideal for production of high purity pharmaceuticals) and cheaper and simpler downstream processing and purification (Kim et al., 2008). Additionally, the use of suspension cultured cells as expression system could reduce heterogeneity of the protein and of the N-glycan, owing to the uniformity in the size and type of cells (Liénard et al., 2007; De Muynck et al., 2009). Moreover, the system is rapid, because there is no need to regenerate and characterize transgenic plants, such that productive cell lines can be generated within a few months (Shaaltiel et al., 2007; Aviezer et al., 2009). Examples of plant suspension culture-produced biopharmaceuticals include Dow Agroscience's purified injectable Newcastle disease virus (NDV) vaccine for chickens, which has been approved by the USDA Center for Veterinary Biologics (http://www.dowagro.com/animalhealth) and Protalix's recombinant human glucocerebrosidase for the treatment of Gaucher's disease (http://www.protalix.com, Shaaltiel et al., 2007). Though cheaper, safer, easier to manipulate and more rapid than most

established conventional systems, suspension culture is still not the best production platform the plant system does offer, as the overall yield and usability is somewhat limited by the diminishing level of recombinant protein during the late stationary phase due to increased proteolytic activity (Corrado and Karali, 2009). Besides, the system is still limited to small number of well-characterized plant cell lines (such as tobacco, rice carrot, or *Arabidopsis*) (Breyer et al., 2009).

2.4. Transient expression systems

The transient production platform is perhaps the fastest and the most convenient production platform for plant molecular farming (Rybicki, 2010). The systems, which are mainly used for quick validation of expression constructs, are now being used routinely for the production of considerable amounts of proteins within a few weeks (Vézina et al., 2009). These include the following methods.

2.4.1. Agroinfiltration method
The agroinfiltration method, which was developed by Kapila et al. (1997), involves infiltration of a suspension of recombinant *Agrobacterium tumefaciens* into tobacco leaf tissue, which facilitates the transfer of T-DNA to a very high percentage of the cells, where it expresses the transgene at very high levels without stable transformation, as in the case of transgenic crops. This method has now been developed into a very rapid, high-yielding transient expression strategy for producing clinical grade bio-pharmaceuticals (Vézina et al., 2009; Pogue et al., 2010; Regnard et al., 2010).

2.4.2. Virus infection method
The virus infection method is dependent on the ability of plant viruses such as tobacco mosaic virus (TMV) and potato virus X (PVX) to be used as vectors to deliver foreign genes into plants, without integration (Porta and Lomonossoff, 2002). Both expression platforms infect tobacco plants and then transiently express a target protein in the plant tissue. Meanwhile, Varsani et al. (2006) were able to successfully obtain protein yield as high as 17% of the total protein, using this expression method. However, as with other fresh plant-based production systems, the recombinant protein has to be processed immediately to prevent tissue degradation and of course protein instability. This represents the only minor drawback of this system. Nonetheless, a pharmaceutical company, Large Scale Biology Corporation has adopted the system for the production of idiotype vaccines for the treatment of B-cell non-Hodgkin's lymphoma, which have successfully passed the phase I clinical testing (McCormick et al., 2008).

2.4.3. The magnifection technology
Both the viral vector-based expression system and *Agrobacterium*-mediated method are limited in their inability to achieve high-level co-expression of two or several polypeptides necessary for the assembly of heterooligomeric proteins (Giritch et al., 2006). Hence the evolution of a new robust transient expression system known as MagnICON®technology, which was developed by Icon Genetics, by removing the coat protein (responsible for systemic movement) of the noncompeting virus strains and the systemic delivery of the resulting viral vectors to the entire plant using *Agrobacterium* as the vehicle of delivery and primary infection (Gleba et al., 2005). This method has not only improved infectivity but also remarkably enhanced amplification and ultimately lead to high-level co-expression of several polypeptides, which were able to assemble functional heterooligomeric proteins at much increased levels up to 100-fold, including a full sized IgG (Giritch et al., 2006) and *Yersinia pestis* antigens fusion protein F1–V (Rosales-Mendoza et al., 2010).

Other advanced viral vectors are currently being designed by various platforms such as, Icon Genetics, Kentucky BioProcessing, Fraunhofer, and host of others, to facilitate ease of manipulation,

wider host range, speed, safety, low cost and high quality and yield of pharmaceutical proteins required for clinical development (http://www.kbpllc.com, Komarova et al., 2010; Pogue et al., 2010; Rybicki, 2009).

3. Limitations and optimization of plant production systems

In spite of the vast advantages of the plant production systems over the established systems (Buonaguro and Butler-Ransohoff, 2010; Obembe, 2010), there exist however, some limitations that make them have comparatively lower social acceptance. The problem of low yield (which can result from low-level transcript expression or the instability of the recombinant protein) and non-mammalian glycosylation are the two major challenges that militate against the full exploitation of plants as alternative bioreactors to the mammalian cell culture, especially. The challenge of choosing the most suitable host plant for any specific biomolecule as well as downstream processing is also being given due attention.

3.1. The problem of low yield

3.1.1. Optimizing transcript expression
In order to optimize transcript expression, the general strategy is the use strong and constitutive promoters, such as the cauliflower mosaic virus 35S RNA promoter (CaMV 35S) and maize ubiquitin-1 promoter (*ubi*-1), for dicots and monocot, respectively (Fischer et al., 2004). Organ- and tissue-specific promoters are also being used to drive expression of the transgenes (vaccine antigen *HBsAg M*, murine single chain variable fragment (scFv) G4 and human interferon-α) in the tissue or organ like the tuber, the seed and the fruit (He et al., 2008; De Jaeger et al., 2002; Masumura et al., 2006). This tissue-specific expression therefore prevents accumulation of the recombinant protein in the vegetative organs, which might negatively affect plant growth and development. Additionally, inducible promoters, whose activities are regulated by either chemical or external stimulus, may equally be used to prevent the lethality problem (Corrado and Karali, 2009), as it is being used in cell suspension cultures (Nara et al., 2000; Peebles et al., 2007). Besides, transcription factors can be used as boosters for the promoters to further enhance the expression level of the transgenes (Yang et al., 2001). Moreover, it has been recently found that the terminator of the heat-shock gene of *Arabidopsis thaliana* shows an increase in transcription of a foreign gene by four times (Nagaya et al., 2010).

Furthermore, the expression constructs can also be designed to ensure transcript stability and translational efficiency. This involves, for instance, the removal of the native 5′ and 3′ untranslated regions from the foreign gene and introducing 5′ untranslated leader sequence of the tobacco mosaic virus RNA, rice polyubiquitin gene RUB13, alfalfa mosaic virus or tobacco etch virus in the expression construct, all of which have been separately shown to significantly enhance the expression levels of transgenes (Lu et al., 2008; Sharma et al., 2008). In addition to these leader sequences, the expression cassette design can be such that the false AU-rich sequences in the 3′ untranslated regions that may act as splice sites are removed or modified, to ensure transcript stability (Mishra et al., 2006). Besides, the transcript stability can be ensured by co-expressing the gene of interest and a suppressor of RNA silencing (Voinnet et al., 2003). It is also established that each organism exhibits biased codon usage, such that it might be important to adapt the coding sequence of the heterologous gene to that of the host plant in order to optimize translation efficiency (Liénard et al., 2007;). In this regard, the translational start-site of the heterologous protein is modified to match with the Kozak consensus for plants (Kawaguchi and Bailey-Serres, 2002) or by using the sequence GCT TCC TCC after initiation codon, or ACC or ACA before it (Sharma and Sharma, 2009). The codon modification, however, should be empirically determined rather than

predicted because of the variation in the levels of transgene expression in the same system, using the same construct (Rybicki, 2009). To this end, codon combinations (A/G)(a/c)(a/g) AUG and (A/G)(u/C)(g/C) AUG have been reported to be optimum for enhanced translational activity in Arabidopsis and rice, respectively (Sugio et al., 2010). This variation in the expression of the transgene may be due to position effect, copy number of the transgene or gene silencing. With respect to position effect, expression cassettes can now be designed to contain the nuclear matrix attachment regions (MAR), which are regulatory sequences that ensure the placement of the transgene in suitable regions for mobilizing transcription factors to the promoters (Streatfield, 2007). Besides, the problem of position effect can be avoided by targeting the transgene into the plastids (Cardi et al., 2010). For optimizing the generation of single-copy transgenics, the strategies that have been used include the use of specific genetic elements, including the cAMP response elements (CREs), for co-transfer with transgene in the T-DNA (De Paepe et al., 2009). Additionally, a new technology, which consists in the construction of genetically autonomous artificial minichromosomes has been described as providing infinite possibilities with several enormous advantages including gene stability; owing to the absence of gene silencing and position effect (Ananiev et al., 2009).

3.1.2. Optimizing protein's stability

For optimizing the stability of recombinant protein, which has been considered as the single most important factor limiting the yields in molecular farming (Schillberg et al., 2005) certain subcellular targeting might be needed. Not only does subcellular targeting enhance protein stability, but it also determines the type of the associated protein processing, and by adding fusions and affinity tags, it can be used to improve downstream processing of isolation and purification (Fischer et al., 2004). Proteins can be targeted to the secretory pathway by an N-terminal signal peptide, which is cleaved off for the release of the protein into the endoplasmic reticulum (ER). Nonetheless, full-size immunoglobulins that are addressed to the secretory pathway have been shown to accumulate at higher levels and easily recovered when fused with to a stabilizing partner (Benchabane et al., 2008; Floss et al., 2009). Most plant-derived recombinant proteins produced so far have been secreted into the apoplastic space of the cell wall (Streatfield, 2006), and have been reported to accumulate at levels up to 100-fold greater than in the cytosol (Schillberg et al., 1999). Recent evidence however suggests that the tomato cathepsin D inhibitor (SlCDI) has the potential to be used as an in-built protein-stabilizing agent for the protection of cytosol-targeted recombinant proteins in plants (Goulet et al., 2010).

The stability and accumulation of recombinant protein could be further increased by targeting to and retaining in other subcellular compartments of the secretory pathways, such as the ER (Conrad and Fiedler, 1998). This is accomplished by appending of SEKDEL ER retention signal to the C-terminus of the protein (Rademacher et al., 2008) or by using the N- or C-terminus fusion of γ-zein signal, which has been found to be more efficient and more economical for production of recombinant proteins (Mainieri et al., 2004; Torrent et al., 2009a, 2009b).

Proteins that do not require post-translational modification like glycosylation for their activity can be targeted to the chloroplast, or better still the foreign gene can be used to transform chloroplast directly, with highly enhanced protein accumulation. Moreover, post-translational modifications of the ER lumen can also be avoided by expressing the protein as translational fusion with oleosin protein, which target the expression of the foreign protein to oil bodies of the seeds (Moloney and Siloto, 2004), which in addition facilitates easy purification of the protein (as discussed later under cost of downstream processing). Besides, proteins can be targeted to membrane surfaces and protected from cytosolic degradation without necessarily passing through the ER lumen by fusing the C-terminal

region of a tail anchored (TA) protein (Maggio et al., 2007). Other subcellular compartments like the protein-storing vacuoles are also now being explored for accumulating recombinant protein, as it has been observed in rice seed endosperm (Yang et al., 2003). Extracellular proteolytic cleavage is another important factor for consideration in plant-based production of pharmaceuticals (Komarnytsky et al., 2006; De Muynck et al., 2009). Komarnytsky et al. (2006) were able to achieve a threefold increase in expression of an antibody when co-expressed with a secreted form of the Bowman–Birk serine peptidase inhibitor than when co-expressed with a cytosolic form of this inhibitor. The addition of gelatin as a substitution substrate for peptidases was also found to increase antibody levels to as high as 300% in tobacco rhizosecretion (Drake et al., 2003). In addition, suspension cells culture media formulations that are rich in peptidase inhibitor might provide safe in vitro environments for human IgG, especially in tobacco. Better still, it would be worthwhile to develop extracellular peptidase-free host plants or to engineer antibodies displaying higher resistance to peptidases while maintaining their activity (De Muynck et al., 2010).

3.2. The glycosylation challenge

Glycosylation is the covalent linkage of sugar moieties to proteins, in order to enhance their folding, biological activity, solubility and bioavailability (Liénard et al., 2007). In plant, glycosylation occurs in the secretory pathway in the ER and the Golgi apparatus. However, there are differences in the glycosylation patterns between the plants and animals, with respect to N-glycan composition. Plants add $\alpha(1,3)$ fucose and $\beta(1,2)$ xylose residues to the N-glycan of their glycoproteins, whereas mammals add $\alpha(1,6)$ fucose moieties, glucose and sialic acid residues to the N-glycan. Hence, to prevent the problem of immunogenicity and allergic reactions that these differences could potentially cause, when plant-derived therapeutic animal proteins are administered to humans (Krapp et al., 2003), there is need to engineer the plant to perform authentic human N-glycosylation. There are a number of strategies that have been used to modify the N-glycosylation pattern in the plants (Gomord et al., 2010). These include the fusion of the ER-retention signal KDEL, which restricted glycosylation of plant-derived antibodies to only high mannose-type N-glycan (Floss et al., 2009), even though, however, such antibodies with high mannose N-glycan have been reported previously to be unstable after injection into mouse (Mainieri et al., 2004). Other strategies that have been used include generation of knock-out mutant lines in Arabidopsis and a moss plant, Physcomitrella patens, that synthesize complex N-glycans lacking immunogenic xylose and fucose epitope (Koprivova et al., 2004; Kang et al., 2008), and modulation of the xylosylated and fucosylated glycan levels, using RNA-interference (Sourrouille et al., 2008), as well as co-expression of an RNA-interference construct with the therapeutic protein (Cox et al., 2006). Also, the knock-in strategy, which involves the expression of a hybrid enzyme obtained through domain fusion of the CST domain of human β-1,4-galactosyltransferase I with β-1,2-xylosyltransferase of Arabidopsis, was shown to cause high-level galactosylation of N-glycans and sharp decrease in the level of N-glycans with xylose and fucose moieties (Vézina et al., 2009). Moreover, synthesis of sialic acid in plant and sialylation of plant-expressed protein has also been reported (Castilho et al., 2010). Hence, it is clear that glycosylation engineering is effective in adapting plant-made antibodies (De Muynck et al., 2010), thus expanding the possibilities for the production of optimally glycosylated proteins with enhanced biological activities for use as human therapeutics (Loos et al., 2010).

3.3. Choice of suitable host plants

The choice of suitable host for deploying the molecular farming technology is crucial for its overall success. In fact, though subtle, this

constitutes the first line of strategy for ensuring efficiency of recombinant protein production. Hence, economic consideration is the key issue in choosing suitable host plants for molecular farming. The important economic factors include total biomass yield, storage properties (facilities), ease of transportation, value of the recombinant protein itself, set-up costs, scale-up and maintenance costs, costs of containment, availability of labor, land area required, length of production cycle, costs of downstream processing and edibility (Fischer et al., 2004; Schillberg et al., 2005). The suitable host should, in addition to economic consideration, be amenable to transformation and regeneration. Hence, the most suitable host plant for a particular recombinant protein has to be determined empirically, based on these highlighted factors, as there is no single perfect system thus far (Schillberg et al., 2005).

In addition to being highly amenable to transformation and regeneration, tobacco also combines most of these economic advantages, such as high biomass yield, high scale-up capacity, non-food/non-feed status (providing containment of transgenic material from contaminating food and feed chain), well-established transformation protocols, all-year round growth and harvesting, and availability of large scale infrastructure for processing (Biemelt and Sonnewald, 2005; Fischer et al., 2004; Stoger et al., 2005a), which explains why it is most preferred for molecular farming of pharmaceutically relevant proteins including antibodies, vaccines and immunomodulatory molecules such as cytokines (De Muynck et al., 2010; Tremblay et al., 2010); many of which are at various stages of development and even commercialization, as in the case of CaroRX™, which is being used for preventing tooth decay (Ma et al., 1998). However, tobacco (except cultivar "81V9, Menassa et al., 2001), contains high amounts of toxic compounds, nicotine and other alkaloids which have to be removed during purification process. Besides, finding a beneficial use for tobacco in place of smoking (which has been banned in public places in most of the developed countries and nowadays in some developing countries) and providing an alternative source of income for tobacco farmers through PMF do not seem to appeal to the existing negative public sentiment against tobacco in the United Kingdom, for instance (Milne, 2008). However, the negative perception of tobacco with respect to its beneficial use might not be an issue, especially when the PMF proteins are not for human consumptions (e.g. for topical applications) and their production are carried out in closed containment facilities, as such tobacco-based production might still maintain its relevance for a long time to come, just as the public comes to term with the fact that this plant can as well be an indirect life-saver. Nevertheless, in the meantime, alternative leafy crops like alfalfa and lettuce are now being explored as suitable hosts for molecular farming (Rosales-Mendoza et al., 2010). Leafy crops, however, generally have a common problem of instability of the expressed proteins, which necessitate that leaves have to be desiccated, or frozen or processed immediately after harvest (Fischer et al., 2004). Additionally, expression in vegetative organs, such as the leaf could affect growth and development of the particular plant.

Seed-based expression seems to be more ideal in this respect, in that, not only does it not affect the growth and development of the plant but it does not need to be frozen or dried or processed immediately, as accumulation of protein in the seed offers protection against proteolytic digestion, as such it allows long-term storage at ambient temperature without the protein losing its activity (Stoger et al., 2002; Nochi et al., 2007). Additionally, seed-based expression platforms are most competitive in applications that require large volume of recombinant proteins per annum (Faye and Gomord, 2010). Moreover, the seed-based system allows oral delivery of vaccine antigens or pharmaceutical proteins for immunization, immunotherapy, and treatment of diseases (Lamphear et al., 2002; Takagi et al., 2005). In this regard, the cereal seeds especially rice and maize are gaining prominence. Maize has many advantages such as having the highest biomass yield among food crops, ease of transformation, and ease of scale-up (Ramessar et al., 2008a), which explains why Prodigene Inc. preferred to use maize for the commercial production of the first recombinant protein, avidin (Hood et al., 1997) and bovine trypsin (Woodard et al., 2003). The Pharma-Planta Consortium has also adopted it for the production of a HIV microbicide (Ramessar et al., 2008b). In addition to sharing many of the advantages of maize, rice is a self-pollinating plant, as such it would have a lower risk of unintended gene flow, which is why Ventria Bioscience has pioneered the production of two rice-derived proteins, human lactoferrin and lysozyme, which have received regulatory approvals, and have already been marketed (http://www.ventria.com, Boothe et al., 2010; Huang et al., 2008; Lau and Sun, 2009). Similarly, ORF genetics Inc. is now exploiting the great potential of cereal seeds (barley) in producing their two commercial products ISOkine™ and DERMOkine™ human-like growth factors, thereby bypassing the use of bacterial or animal cell systems (http://www.orfgenetics.com/, Faye and Gomord, 2010). The advantage of exceptionally high protein content (20–40%) in legume seeds, coupled with the self-pollinating status of soybean and pea has made them candidate platform for accumulating recombinant proteins. Soybean, in particular, has been explored for its efficacy to express a humanized antibody against herpes simplex virus, bovine casein, and a human growth hormone (Philip et al., 2001; Russell et al., 2005; Zeitlin et al., 2009). Soybean was also used to express a functional hypotensive peptide, which reduced the systolic blood pressures of model mice (Yamada et al., 2008).

3.3.1. Cost of downstream processing

The cost of downstream processing of plant-derived recombinant proteins is believed to account for about 80% of the total production costs (Buonaguro and Butler-Ransohoff, 2010; Kusnadi et al., 1998); as such it has been given its well-deserved attention with respect to devising strategies to reduce it to the barest minimum. However, using watery tissues such as tomatoes as the production systems has been advanced to have the potential of lowering this cost, as it is easier to extract from them than dry tissue like cereals (Schillberg et al., 2005; Yano et al., 2010). Additionally, with respect to biosafety concern on containment of the transgenic plants, tomatoes also appear attractive as a host crop, because they are grown in greenhouses. Nowadays, the oil bodies in certain oilseed crops like safflower and rape seeds are now being exploited to ease recombinant protein purification, and minimizing the downstream costs, by using the oleosin fusion technology developed by SemBioSys Genetics (http://www.sembiosys.com/). The strategy involves targeting the recombinant protein to the seed of the oilseed crops as a fusion with oleosin, hence simplifying the extraction of the fusion proteins from the oil bodies and the release of the recombinant protein from its fusion partner, for example in the case of accumulating and purifying biologically active human insulin, Apolipoprotein AI (Milano) and human growth hormone in safflower (Boothe et al., 2010; Nykiforuk et al., 2006; Nykiforuk et al., 2010).

It is envisaged that the advantage of lower cost of production might facilitate the participation of less developed countries in pharmaceutical production in plants (Paul and Ma, 2010). There are indeed several plant-derived recombinant proteins that were earlier thought to be delivered as edible vaccines, which can be eaten directly as fruits (tomatoes, banana) and vegetables (lettuce, carrot), thus eliminating the need for any processing, hence no processing costs (Mason et al., 2002). Banana in particular, was being touted as a candidate host fruit crop for producing edible vaccine, especially for the developing countries, as there would not be need for long-distance transport and satisfying cooling requirements, since it is widely grown in these countries (Biemelt and Sonnewald, 2005). Additional advantages of banana are that of high digestibility and palatability, as such they were earlier gaining public acceptance for

infant vaccination (Kumar et al., 2004, 2005). The suitability of potatoes for oral vaccine production had also generated interest because they can be eaten raw or with just little processing. Potatoes, like seeds, have the advantage of product stability due to the specialized molecular environment in the tuber (Sparrow et al., 2007). Several clinical trials of potato tuber-derived antigens have been successful (Streatfield and Howard, 2003; Walmsley and Arntzen, 2003), however, for the crop to be acceptable for use in oral vaccine administration, there must be some degree of processing, cooking, in order to destroy the endotoxins; a process that will degrade the thermolabile protein products (Sparrow et al., 2007). Due to ethical issues however, the consequential shift in research focus toward the development of heat stable oral vaccines (Rybicki, 2009) is now giving way to the production of plant-derived vaccines that would still be processed further, formulated in a reproducible way and given under supervision so as to ensure reproducible effects (Rybicki, 2010).

As a result of the demand for high containment of transgenic plants these days, safer and more cost-effective alternative plant-based expression platforms are now being sought after, including *Physcomitrella patens*, a moss and green algae, such as *Chlamydomonas reinhardtii*. These two expression systems combine the intrinsic advantage of being amenable to cultivation in bioreactors under controlled conditions (Decker and Reski, 2004, 2008; Franklin and Mayfield, 2004; Hohe et al., 2002; Tran et al., 2009) with the possibility of secreting of recombinant proteins into the medium (thus reducing the extraction and purification costs) (Mayfield and Franklin, 2005), and with a simple transformation procedure and shorter production cycle (Leon-Banares et al., 2004; Schaefer, 2002). *Physcomitrella* has even been engineered to produce a strain that does not add immunogenic $\beta(1,2)$ xylose and $\alpha(1,3)$ fucose (Koprivova et al., 2004), thus eliminating the concern about non-human glycosylation, which is the reason for its adoption by German biotech company, Greenovation Biotech as their production platform, and uniquely branded as bryotechnology (http://www.greenovation.com). Other example of plant-based expression system is the *Lemna* plant, which has very high rate of biomass accumulation per unit of time; doubling in size every 24–48 h (Liénard et al., 2007) and are amenable to transformation and metabolic engineering, for the expression of humanize N-glycans (Cox et al., 2006). Besides, there has been recent revisiting of the rhizosecretion technology, a hydroponic production system, as a suitable, cost-effective production system, which also combines the advantage of confined environments with the flexibility of adding the hydroponic medium directly to a chromatography column for affinity purification, thus allowing simple and rapid production of high purity recombinant protein products (Drake et al., 2009). Three affinity tags commonly used include His$_6$, StrepII and Tandem Affinity Purification (TAP) tag (Terpe, 2003). An elastin-like polypeptide tag has also been optimized for expression and purification of recombinant proteins in plants (Conley et al., 2009).

4. Overview of plant-derived recombinant proteins

4.1. Plant-derived vaccine antigens

A vaccine is an antigenic preparation that improves immunity against a particular disease. The development of non-replicating viral protein subunit was inspired by the intrinsic risks involved in producing large amounts of vaccines through the traditional method of using live attenuated or inactivated viruses (Varsani et al., 2003). Hence, there was a shift to the current production of subunit vaccines in bacteria, yeast, and mammalian cells. However, these conventional expression systems have some peculiar problems. As such, the idea of using plants as a platform for production of vaccines was borne out of its numerous potential advantages over the existing ones, including

the potential for large scale production, production costs reduction and oral delivery option (Streatfield and Howard, 2003; Pujol et al., 2005; Buonaguro and Butler-Ransohoff, 2010) as well as being potential hosts for the production of glycosylated subunit vaccines (Bosch and Schots, 2010). Several vaccines have been expressed in plants, since the first plant-derived vaccine-relevant protein was reported 20 years ago (Rybicki (2009; Tiwari et al., 2009). These include the hepatitis B surface antigen, which has been expressed in transgenic potatoes (Richter et al., 2000), in tomato (He et al., 2008), in banana (Kumar et al., 2005) and in tobacco cell suspension culture (Sojikul et al., 2003), the heat labile enterotoxin B subunit (LTB) of *Escherichia coli*, has ben expressed in potato tubers (Lauterslager et al., 2001), in maize seed (Chikwamba et al., 2002), in tobacco (Rosales-Mendoza et al., 2009), and in soybean (Moravec et al., 2007).

The cholera toxin B subunit (CTB) of *Vibrio cholera* has been expressed in several crops, including tobacco, tomato and rice (Daniell et al., 2001a; Mishra et al., 2006; Nochi et al., 2007), several plant-made vaccines for veterinary purposes including, avian influenza, Newcastle disease, foot-and-mouth disease and enterotoxigenic *Escherichia coli* have been expressed in the plant (Lentz et al., 2010; Ling et al., 2010), and the Hemagglutinin protein of rinderpest has been expressed in pigeon pea and peanut (Satyavathi et al., 2003). Others include the L1 protein of human papillomavirus types 11 and 16 (Biemelt et al., 2003; Maclean et al., 2007; Giorgi et al., 2010), the Norwalk virus capsid protein (Mason et al., 1996), the Hemagglutinin protein from measles virus (Marquet-Blouin et al., 2003) and the H5N1 pandemic vaccine candidate (D'Aoust et al., 2010), all of which have been expressed in one or two of the following plants: tobacco, potato and carrots. Also worthy of note, is the US Defense Department-sponsored research and development of various bio-defense vaccines against anthrax, ricin, plague and haemorrhagic fever virus (Hull et al., 2005; Koya et al., 2005; Santi et al., 2006; Mett et al., 2007).

Plant-produced antigens have been reported to induce immune responses, conferring protection against challenge in mouse model systems (Satyavathi et al., 2003; Streatfield and Howard, 2003). Oral administration of several plant-produced antigens has been reported to induce protective immune response in humans (Thanavala et al, 2005; McCormick et al., 2008) and mice (Daniell et al., 2001b; Tregoning et al., 2005). However, about 100-fold of the vaccine delivered by injection is required for oral administion (Streatfield and Howard, 2003). Notably, Franconi et al. (2006) and Massa et al. (2007) reported that immunization with plant-produced antigen protected the injected mice from tumour challenge with an E7-expressing tumour cell line, while Koya et al. (2005) reported that mice injected and immunized with chloroplast-derived anthrax protective antigen survived anthrax lethal toxin challenge. Also the Gal/GalNAc lectin of *Entamoeba histolytica* was found to be highly immunogenic in injected mice (Chebolu and Daniell, 2007). There are several plant-produced vaccine candidates, which are at different stages of clinical trials, as summarized in Table 1. It should be noted that the first veterinary vaccine, the NDV vaccine for poultry developed by Dow AgroSciences, has been approved by the US Department of Agriculture (USDA) (http://www.dowagro.com/animalhealth). As such, the plant-based production processes are able to compete with conventional methods, breaking the limits of current standard production technologies and reaching new frontiers for the plant-based production of pharmaceutical-grade proteins (Buonaguro and Butler-Ransohoff, 2010).

4.2. Plant-derived antibodies

Antibodies or immunoglobulins (IgGs) are serum proteins that bind to target molecules with high specificity and are widely used for prevention, detection and treatment of diseases. Up till recently, monoclonal antibodies were only being used to treat diseases such as arthritis, cancer, immune and inflammatory diseases. Recombinant

Table 1
Plant-derived pharmaceuticals in clinical stages of development or on market. Information from Basaran and Rodríguez-Cerezo (2008), Kaiser (2008), Key et al. (2008), Spök et al. (2008), Sharma and Sharma (2009), Lau and Sun (2009), Obembe (2010), Faye and Gomord (2010). Updated from company websites.

Product	Disease	Plant	Clinical trial status	Company	Source: URL/academic
Vaccines					
Hepatitis B antigen (HBsAg)	Hepatitis B	Lettuce	Phase I	Thomas Jefferson University, USA	Streatfield, 2006
		Potato	Phase II	Arizona State University	
Fusion proteins, including epitopes from rabies	Rabies	Spinach	Phase I completed	Thomas Jefferson University, USA	www.labome.org/expert/usa/.../hilary-koprowski-233492.html
Cancer vaccine	Non-Hodgkin's lymphoma	Tobacco	Phase II	Large Scale Biology[a], USA	http://www.gmo-safety.eu/article/483.pharma-plants-status-report.html
Vibrio cholerae	Cholera	Potato	Phase I	Arizona State University	Tacket, 2005
Heat-labile toxin B subunit of *Escherichia coli*	Diarrhea	Maize	Phase I	ProdiGene[b], USA	Tacket, 2005
		Potato	Phase I	Arizona State University	
Capsid protein Norwalk virus	Diarrhea	Potato, Tomato	Phase I	Arizona State University	Khalsa et al., 2004
Antigen	Feline parvovirus (Dogs)	Tobacco	Advanced	Large Scale Biology[a], USA	http://www.lsbc.com
Antigen	Papilloma virus (Rabbit)	Tobacco	Early	Large Scale Biology[a], USA	http://www.lsbc.com
HN protein of Newcastle disease virus	Newcastle disease (Poultry)	Tobacco suspension cells	USDA Approved	Dow Agro Sciences, USA	http://www.dowagro.com/animalhealth
Viral vaccine mixture	Diseases of horses, dogs, and birds	Tobacco suspension cells	Phase I	Dow Agro Sciences, USA	http://www.dowagro.com/animalhealth
Poultry vaccine	Coccidiosis infection	Canola	Phase II	Guardian Biosciences, Canada	Basaran and Rodríguez-Cerezo (2008)
Gastroenteritis virus (TGEV) capsid protein	Piglet gastroenteritis	Maize	Phase I	ProdiGene[b], USA	Basaran and Rodríguez-Cerezo (2008)
H5N1 vaccine candidate	H5N1 pandemic influenza	Tobacco	Phase I	Medicago, USA	http://www.medicago.com
Antibodies					
CaroRX	Dental caries	Tobacco	EU approved as medical advice	Planet Biotechnology, USA	http://www.planetbiotechnology.com/
DoxoRX	Side-effects of cancer therapy	Tobacco	Phase I completed	Planet Biotechnology, USA	http://www.planetbiotechnology.com/
RhinoRX	Common cold	Tobacco	Phase I completed	Planet Biotechnology, USA	http://www.planetbiotechnology.com/
Fv antibodies	Non-Hodgkin's lymphoma	Tobacco	Phase I	Large Scale Biology, USA	http://www.lsbc.com[a]
IgG (ICAM1)	Common cold	Tobacco	Phase I	Planet Biotechnology, USA	http://www.planetbiotechnology.com/
Antibody against hepatitis B	Vaccine purification	Tobacco	On market	CIGB, Cuba	Kaiser, 2008
Therapeutic human proteins					
Gastric lipase, Merispase®	Cystic fibrosis	Maize	On market	Meristem Therapeutics France	http://www.meristem-therapeutics.com
α-Galactosidase	Fabry disease	Tobacco	Phase I	Planet Biotechnology, USA	http://www.planetbiotechnology.com/
Lactoferon™ (α-interferon)	Hepatitis B and C	Duckweed	Phase II	Biolex, USA	http://www.biolex.com/
Fibrinolytic drug (thrombolytic drug)	Blood clot	Duckweed	Phase I	Biolex, USA	http://www.biolex.com/
Human glucocerebrosidase (prGCD)	Gaucher 's disease	Carrot suspension cells	Awaiting USDA's approval	Protalix Biotherapeutics, Israel	http://www.protalix.com/glucocerebrosidase.html
Insulin	Diabetes	Safflower	Phase III	SemBioSys, Canada	http://www.sembiosys.com/
Apolipoprotein	Cardiovascular	Safflower	Phase I	SemBioSys, Canada	http://www.sembiosys.com/
Nutraceuticals					
ISOkine™, DERMOkine™	Human growth factor	Barley	On market	ORF Genetics	http://www.orfgenetics.com/
Human intrinsic factor, Coban	Vitamin B12 deficiency	*Arabidopsis*	On market	Cobento Biotech AS	http://www.cobento.dk/default.asp?id=76
Human lactoferrin	Anti-infection, anti-inflammatory	Rice	Advanced, on market as fine chemical	Ventria, USA	http://www.ventriabio.com/
Human lysozyme	Anti-infection, anti-inflammatory	Rice	Advanced, on market as fine chemical	Ventria, USA	http://www.ventria.com/
Immunosphere™	Food additive for shrimps	Safflower	Marketing expected for 2010	SemBioSys, Canada	http://www.sembiosys.com/

[a] LSBC filed bankruptcy in 2006.
[b] Prodigene has winded up activity.

antibodies have now been found to provide passive immunization against pathogens, as such are potentiated as promising alternatives to combat infectious diseases, especially in the face of increasing microbial resistance to antibiotics and the emergence of new pathogens (Casadevall, 1998). Though, there is increasing market demand, however, the prevailing high cost of production is preventing their successful introduction into the health market, as therapy of infectious diseases. These opposing factors, thus, call for cost-effective alternatives for large scale production (Stoger et al., 2005b), as bulk production in the established production system- the mammalian cells, presently poses difficult challenge, with respect to the complexity of the product and the different processes involved, such as post-translational modifications and proteolysis. Plants do not only provide cheaper production platforms, as plant-derived antibodies would cost just 0.1–1% of the production cost of mammalian culture and 2–10% of microbial systems (Chen et al., 2005), but also can also

assemble complex multimeric antibodies (Conrad and Fiedler, 1994), as such can be engineered for the production of diverse tailor-made pharmaceutical proteins. Since the first recombinant antibodies were expressed in plants in 1989 (Hiatt et al., 1989), different moieties ranging from single chain Fv fragments (ScFvs, which contain the variable regions of the heavy and light chains joined by a flexible peptide linker) to Fab fragments (assembled light chains and shortened heavy chains), small immune proteins (SIP), IgGs, and chimeric secretory IgA and single-domain antibodies have been expressed as well (Ismaili et al., 2007; Xu et al., 2007).

There are two main approaches that are being employed to produce biologically active whole antibodies in plants. The first is cross-pollination of individually transformed plants expressing light or heavy chains, resulting in high yield which reaches 1 to 5% of total plant protein (Hiatt et al., 1989; Ma et al., 1994). The second approach involves co-transformation of the heavy and light chain genes on a single- (During et al., 1990), two- (Villani et al., 2008) or more expression cassettes (Nicholson et al., 2005). One of such plant-derived antibodies, a secretory antibody against a surface antigen of *Streptococcus mutans* was actually found to be as effective as the original mouse IgG, in protecting against *S. mutans* colonization on teeth, (Ma et al., 1998). The antibody has since been developed further by Planet Biotechnology, Inc. into a clinical product, CaroRX™, which has recently received EU approval to be used as medical advice for the prevention of oral bacterial infection that contributes to dental caries (Kaiser, 2008). Recently, a HIV-specific monoclonal antibody, which was produced in maize seeds, was found to be as active as its Chinese hamster ovary-derived counterpart, in terms of antigen-binding activity (Ramessar et al., 2008a, 2008b). There are five different plant-derived monoclonal antibodies presently being tested in the clinical trials, as listed in Table 1. The sixth one, Avicidin was withdrawn from the trials because of several side effects suffered by the participants, even though it showed promising results against advanced colon and prostate cancer (Ma et al., 2005). The first plant-made scFv monoclonal antibody, used in the production of a recombinant hepatitis B virus vaccine, has been commercialized in Cuba (Pujol et al., 2005).

4.3. Therapeutic and nutraceutical proteins

The first therapeutic human protein to be expressed in plants was a human growth hormone (Barta et al., 1986). In 1990, human serum albumin, which is normally isolated from blood, was produced in transgenic tobacco and potato for the first time (Sijmons et al., 1990). Since then, several human proteins have been expressed in the plants. These include epidermal growth factor (Wirth et al., 2004; Bai et al., 2007), α-, β- and γ-interferons, which are used in treating hepatitis B and C (Sadhu, and Reddy, 2003; Zhu et al., 2004; Arlen et al., 2007), erythropoietin, which promote red blood cell production (Weise et al., 2007; Musa et al., 2009), interleukin, which is used in treating Crohn's disease (Elias-Lopez et al., 2008; Fujiwara et al., 2010), insulin, which is used for treating diabetes (Nykiforuk et al., 2006), human glucocerebrosidase, which is used for the treatment of Gaucher's disease in genetically engineered carrotcells (Shaaltiel et al., 2007) and several others. Also contained in Table 1 is the list of some of these therapeutics, which are at various stages of clinical trials or at the verge of being commercialized.

Antimicrobial nutraceutics, such as human lactoferrin and lyso-zymes, have now been successfully produced in several crops (Huang et al., 2008; Stefanova et al., 2008), and now commercially available, only as fine chemicals (Table 1). Cobento Biotechnology, Denmark has just recently received approval for its *Arabidopsis*-derived human intrinsic factor (Key et al., 2008), which is to be used against vitamin B12 deficiency and now commercially available as Coban (Sharma and Sharma, 2009). Other nutraceutical products at various stages of development are listed in Table 1.

4.4. Non-pharmaceutical plant-derived proteins

The non-pharmaceutical plant-derived proteins or industrial proteins, most of which are enzymes, including avidin, trypsin, aprotinin, β-glucuronidase, peroxidase, laccase, cellulase and others listed in Basaran and Rodríguez-Cerezo (2008) are now on the market. The molecular farming of cell-wall deconstructing enzymes such as cellulases, hemicellulases, xylanases and ligninases, in particular, holds great promise for the biofuel industry, with respect to the production of cellulosic ethanol (Sticklen, 2008; Mei et al., 2009; Chatterjee et al., 2010), which was estimated to have the potential of reducing greenhouse gas emissions by 100% compared to gasoline (Fulton et al., 2004). Other non-hydrolytic proteins, such as the cell wall disrupting carbohydrate binding modules of cell wall deconstructing enzymes, and the cell wall loosening proteins, the expansins, which have been implicated in enhancing the efficiency of wall degradation by disrupting the different polysaccharide networks, thereby allowing increase accessibility of the hydrolytic enzymes to the substrate, have been previously demonstrated to alter cell wall structure (Obembe et al., 2007a; Obembe et al., 2007b), and as such have been recommended as candidate PMF proteins for the biofuel industry (Obembe, 2010).

Other potential non-pharmaceutical plant-derived technical proteins that are being explored and optimized for production include biodegradable plastic-like compounds such as polyhydroxyalkanoate (PHA) copolymers, Poly (3-hydroxybutyrate) (PHB) and cyanophycin (Conrad, 2005; Matsumoto et al., 2009). It should be noted that thus far, only few plant-derived pharmaceuticals have been approved, and fewer still are commercially available, majorly because of biosafety concerns and stringent governmental regulations with respect to field trials, good manufacturing practice (GMP) standards and pre-clinical toxicity testing.

5. Biosafety and regulatory issues

The general public concern about the potential health and environmental risks associated with the PMF crops (not the products) is being viewed at two levels; in that, not only are they engineered to accumulate all sorts of proteins with medicinal properties at very high concentration, which may affect the host plants (Badri et al., 2009), but also that their biologically active products are meant to elicit physiological responses in humans and in animals (Spök, 2007; Spök et al., 2008). Additionally, there are specific concerns, including the risks of (i) co-mingling with food/feed crops and the resultant economic risks to farmers and food industry, (ii) transgenes spread by pollen, seed or fruit dispersal, (iii) unintended exposures to non-target organisms, particularly birds, insects and soil microorganisms, and (iv) horizontal gene transfer by asexual means (Commandeur et al., 2003; Andow et al., 2004; Wisner, 2005).

Like for the GM food and feed crops, several regulations are being developed, to increase the biosafety of the plant bioreactors, even though, one knows that, there is no fool-proof system, as there might be some elements of human errors and natural accidents, which are beyond control.

5.1. Problem of co-mingling and contamination of the food/feed chain

The problem of co-mingling and contamination of the food/feed chain is being prevented by the use of non-food and non-feed crops, such as tobacco, lemna, Arabidopsis, microalgae or mosses (Cox et al., 2006; Decker and Reski, 2008; Franklin and Mayfield, 2004; Tremblay et al., 2010). However, when it is desirable to use food or feed crops, as in the case of monoclonal antibodies and oral vaccines, strict physical agronomic confinement and containment strategies are being applied to avoid co-mingling. These include growing in small restricted and isolated areas, remote from the agricultural crops to avoid mechanical

mixing (Ma et al., 2005; US Department of Agriculture, 2006; Spök et al., 2008), crop destruction and post-planting field cleaning, planting at different periods to ensure harvesting at different periods from other crops intended for use as food and feed (Spök, 2007), the use of non-commercial varieties or plant containing visible marks (Biemelt and Sonnewald, 2005), prohibition of trade in seeds and viable plants (Spök et al., 2008), and the use of dedicated machineries and facilities (US Department of Agriculture, 2006). As it is very difficult to avoid admixtures of GM and non-GM crops, even with confinement (USDA/APHIS, 2006b), threshold limits of accidental contaminations are now being introduced, for example in Europe, 0.5% presence of GM in non-GM or feed is allowed (European Parliament, 2003) and for non hazardous, non-pharmaceutical plant-made products, the threshold limit of contamination is set at 0.9% (Spök, 2007).

5.2. The problem of gene spread and unintended exposures

The biosafety strategies being used to address the problem of gene spread and unintended exposures of the plant bioreactors, irrespective of the status (food or non-food crop), include the use of closed isolated containment facilities, such as greenhouses (Menassa et al., 2001), glasshouses, hydroponics (Commandeur et al., 2003; Drake et al., 2009) and plant cell suspension cultures (www.protalix.com) (Franconi et al., 2010; Plasson et al., 2009), the use of biological containment, such as self-pollinating species (cleistogamous lines) (Leflon et al., 2009), chloroplast transformation (Svab and Maliga, 2007), the use of male-sterile transgenic plants (Gils et al., 2008), the use of sexually incompatible crop with wild relatives, the production of non-germinating seeds or non-sprouting tubers/bulbs (through the use of a technology known as "Genetic use restriction technologies (GURTs)" (Hills et al., 2007). Other strategies being pursued to minimize the gene spread and exposure to other organisms include cytoplasmic male sterility (Chase, 2006), engineered parthenocarpy and apomixis (Rotino et al., 2005; Sandhu et al., 2009), transgene excision (Gidoni et al., 2008), engineering the plants for organ- or tissue-specific expression of the transgene (for example in the fruits) (He et al., 2008), and the use of inducible promoters that respond to external stimuli or chemicals can be exploited to regulate gene expression at some point after harvest (Corrado and Karali, 2009), especially as it has been shown that certain plant-derived insecticides and biopharmaceutical proteins can remain stable in the soil for a long period (Basaran and Rodríguez-Cerezo, 2008). Another strategy being deployed for curtailing the effect of gene spread is transgenic mitigation, which does not by itself prevent transgene flow from transgenic crops to nontransgenic crops or wild relatives, but 'mitigates' the effects of such gene flow, i.e. its goal is to prevent the establishment of the transgene in volunteer populations or in populations of wild relatives if hybridization can occur (Gressel and Valverde, 2009).

5.3. Risk of horizontal gene transfer

The risk of horizontal gene transfer from plants to microbes, especially in the case of using antibiotic resistance genes, is generally believed to be extremely low, as there has been no report of such incidence to date. More so, it is widely known that plants, including the edible ones, naturally harbor loads of bacteria with antibiotic-resistant genes (Nielsen et al., 1998).

It should be noted that most of the aforementioned biosafety strategies have been motivated by regulatory policies and guidelines of the different countries involved in the research, development and industrial applications of the PMF technologies. However, it has been admitted that the enforcement of the regulations is still leaky, as it is difficult to monitor all the different stages of development and processing of the plant-derived products, as such there exists some laxities in the level of compliance on the part of the operators, which go undetected by the regulatory agencies. The civil society now perceives the regulatory guidelines on GM crops in general as being relaxed or compromised, which explains why some unapproved GM foods still manage to find their ways to the dining tables in the US and most unexpectedly in Europe. This calls for stricter compliance with good manufacturing practice standards, all through the stages of development, in order to ensure the delivery of clinical grade plant-derived products.

6. Concluding remarks

No doubt the PMF industry is here to stay, considering the quantum of advances that have been made in the past decade alone. Though, there had been some disappointing incidence at some points, which led to the lull in the industry, and even to the folding up of some of the big players, including Prodigene, we do share the same sentiment with Kaiser (2008) that exciting times are now returning to the industry, as a number of new players are now picking up interest and entering into the field. One such player is the multinational company Bayer, which is collaborating with a number of biotech companies and has recently bought the German company, Icon Genetics that developed the MagnICON technology. Also worth mentioning, is the research program of the EU-funded Pharma-Planta consortium, which is devoted to developing and ultimately producing recombinant pharmaceuticals in plants, for humanitarian purpose! With their recent successful development of a cost-effective seed-based production technology for a vaginal protection microbicide to prevent heterosexual HIV transmission, the stage in now set for the deployment of the same technology to produce other therapeutic bio-molecules on a large scale, which could be made more widely available and cheaper than presently possible (Ramessar et al., 2008a, 2008b). In 2009, another International Research Group (consisting of Universities, Research Institutes and new biotech companies) successfully accumulated over 1 g/kg of a red algal protein, a lectin known as griffithsin (GRFT-P) in *Nicotiana benthamiana* leaves (O'Keefe et al., 2009). This protein, in addition to exhibiting broad-spectrum activity against a wide range of HIV strains and similar viruses causing sexually transmitted infections, represents the most potent HIV entry inhibitor to date (Zeitlin et al., 2009; O'Keefe et al., 2009). Having met all the requirements to be used as a microbicide, GRFT-P is now being validated as a candidate protein microbicide for clinical trials (O'Keefe et al., 2009) and it is hoped that the recent results on efficacy dilution of candidate microbicide (Mâsse et al., 2009) would be useful for successful trials. It is envisioned that all of these developments in PMF would begin to attract newer stakeholders to join in, especially for clinical development of large volume biologics, which are presently very expensive to manufacture with the conventional manufacturing practice. Most of all, the overall prospects of plant-based molecular farming industry will depend on improved public perception of the technology and the products, especially as the industry improves its level of compliance with the regulations, and as there are many successful clinical trials and approvals of the first set of these plant-derived human pharmaceuticals. It is also envisioned that the public perception would be all the more positive with the recent European Union's preparation for "pharmed drugs" (Gilbert, 2009; Rybicki, 2010).

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