Review

Biosafety considerations associated with molecular farming in genetically modified plants

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The production in genetically modified plants of recombinant proteins for pharmaceutical or industrial use, also referred to as "plant molecular farming", deserves increasing interest due to its potential advantages. However, this type of application of genetic engineering also raises some biosafety concerns, in particular regarding aspects such as transgene spread in the environment or accidental contamination of the food and feed chains. This review presents the current state of the art of this sector, discusses some relevant regulatory issues and outlines important scientific aspects that should be considered during the safety assessment of genetically modified plants grown for this purpose. In particular, it addresses general strategies as well as specific potential containment measures that could be applied to limit the potential environmental and human health impacts linked to plant molecular farming.

Key words: Biopharming, biosafety, confinement, containment, genetically modified organism, plant molecular farming, plant-made industrials, plant-made pharmaceuticals.

INTRODUCTION

Plant Molecular Farming (PMF) consists of using trans-genic plants as production platforms for the synthesis of compounds for pharmaceutical or industrial purposes. PMF has been presented as a convenient way to produce molecules of interest on a large scale at low costs. Other benefits associated with the use of plants are a rapid scaling up, convenient storage of raw material and less concern over human pathogen contamination in preparation (Raskin et al., 2002; Twyman et al., 2003).

As with current Genetically Modified (GM) plants, all transgenic plants intended for molecular farming must go through a thorough health and environmental risk assessment before they can be used. In that respect, PMF raises novel questions that could trigger a need for specific biosafety considerations. In this review, we outline the main challenges linked to the assessment of the environmental and health risks associated with PMF and discuss several options available for risk manage-ment.

The potential societal and ethical issues linked to plant molecular farming are outside the scope of this review, as well as aspects related to quality, purity or efficacy of the products.

PLANT-BASED SYSTEMS FOR MOLECULAR FARMING

Pharmaceutical and industrial applications of GM plants deserve a growing interest. The production of high-value recombinant proteins in plants appears indeed to have several potential advantages compared to alternative production platforms currently used such as microbial fermentation or culture of mammalian cell lines (Daniell et al., 2001a; Twyman et al., 2005). These advantages include:

i. Rather straightforward and cost-effective culture and processing technology in plants.
ii. Ability to perform most post-translational modifications required for giving functional proteins (Gomord et al., 2005).
iii. Increased safety to human health of products synthesized in plant systems since the risks arising from

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the contamination with human pathogens or toxins are minimized.
iv. Purification processes that can be avoided (when the plant tissue containing the recombinant protein is used directly for product delivery) or greatly facilitated (when recombinant molecules can be targeted or expressed directly into certain intracellular compartments).

A great diversity of plants is currently being used for PMF. This includes food/feed plants like alfalfa, clover, lettuce, maize, rice, wheat, barley, soybean, oilseed rape, pea, potato and tomato, non-food plants like tobacco, *Arabidopsis* as well as duckweed, mosses and microscopic algae (Howard, 2005; Ma et al., 2003; Streatfield and Howard, 2003; Fischer et al., 2004; Goldstein and Thomas, 2004).

Some proteins produced by PMF are already on the market: avidin (Hood et al., 1997), ß-glucuronidase (Witcher, 1998), trypsin (Woodard et al., 2003) and aprotinin (Howard, 2005). More than 500 recombinant pharmaceutical products are believed to be in development worldwide, including agents directed against cancer, infectious diseases and a variety of important medical conditions such as monoclonal antibodies (Daniell et al., 2001a). Plant-based platforms are also used for producing subunit vaccines, some of them being in clinical trial stage (Ma et al., 2005a). In addition to vaccines meant for humans, plant-based vaccines and antibodies are being developed for use in animal health as well (Floss et al., 2007).

The production of heterologous recombinant proteins in plants offers also a range of potential applications in the field of industrial products, although their development does not appear to be as advanced as for plant-made pharmaceuticals (PMPs) (Hood, 2002). Plant-made industrial products (PMIs) currently in the pipeline include enzymes that can be used in detergents, bio-plastics, secondary metabolites (phenolics, glucosinolates, tannins, starches, sugars, fragrances, flavours and alkaloids), fibers or food manufacturing.

Plant-based production platforms appear to be developed and tested mostly in North America. In 2006 Dow AgroSciences LLC received from the United States Department of Agriculture (USDA) the world's first regulatory approval for a plant-made vaccine, a product to protect chickens from the Newcastle disease synthesized from tobacco cells grown in bioreactors (see http://www.dowagro.com/animalhealth/resources/news/20060131b.htm).

In Europe, 41 field trials with such transgenic plants have been notified since 1995 (Table 1). None of these products have already been approved for marketing in Europe but some pharmaceutical proteins (gastric lipase, lactoferrin) have reached clinical development (Ma et al., 2005b). The European Union is also funding two major research programs in the field of PMF:

i. Pharma-Planta, a consortium of 39 research teams from across Europe and South Africa. Its mission is to develop efficient and safe strategies for the production of clinical-grade protein pharmaceuticals in plants and to define procedures and methods for the production of these proteins in compliance with all appropriate regulations (see http://www.pharma-planta.org/).
ii. SmartCell, which brings together 14 leading European academic laboratories and four industrial partners in order to create a novel concept for rationally engineering plants towards improved economical production of high-value compounds for non-food industrial use (http://cordis.europa.eu/fetch?CALLER=FP7_NEWS&ACTION=D&RCN=30444).

REGULATORY BACKGROUND

We provide hereunder a brief overview of the regulations and guidance's pertaining to plant molecular farming focusing on the situation in the United States and the European Union.

In the US, the basic institutional structure for regulating biotechnology products is the Coordinated Framework for Regulation of Biotechnology created in 1986 (see http://usbiotechreg.nbii.gov). In case of PMF, the USDA's Animal and Plant Health Inspection Service (APHIS) is the main agency involved in the regulatory process pertaining to the cultivation while the Food and Drug Administration (FDA) covers the pharmacological and safety aspects when the end product is a pharmaceutical.

GM plants are considered "regulated articles" by APHIS, which means that the use of such plants outside the constraints of physical containment (e.g. in a field) requires an authorization. For most GM plants, this authorization is obtained through a "notification" procedure. Once authorized, the GM plant can, upon successful experimental releases, petition for non-regulated status, meaning that it is no longer subject to APHIS oversight and can then be freely commercialized. This is in fact the case for all the major commercial GM crops currently on the US market. However, with regard to GM plants producing pharmaceutical and industrial compounds, APHIS has adopted strengthened regulatory requirements since 2003.

Accordingly, APHIS requires for these products a more constraining "permit" procedure with specific confinement measures, procedures to verify compliance and ways to enhance the transparency of the permitting system (Federal Register Notice, 2003; NARA, 2005). In addition, no GM plant field-tested under the permit procedure has so far been granted non-regulated status.

In the European Union, unconfined activities for experimental or commercial purposes are regulated by Directive 2001/18/EC on deliberate release of GMOs (EC, 2001). If the GMO is intended for food and/or feed use it falls under the scope of Regulation (EC) 1829/2003 (EC, 2003). Under these two regulatory frameworks, authorizations have to be granted at the EU level, use it falls under falls under the scope of Regulation (EC) 1829/2003 (EC, 2003). Under these two regulatory frameworks, authoriza-

Table 1. Overview of field trials in Europe (1995 - 2009) with transgenic plants used as production platform for pharmaceutical or industrial products.

Crop	Trait/Molecule of interest	Nr of trials
Barley	Thaumatin (sweetener)	1
Barley	Human serum albumin	1
Barley	Growth factor	1
Flax	Biodegradable plastic	1
Maize	Rabies virus G glycoprotein	1
Maize	Dog gastric lipase	6
Maize	Human collagen	2
Maize	Human lactoferrin	2
Maize	Human albumin	1
Maize	Antibodies	1
Rape	Dog gastric lipase	2
Pea	Alpha-amylase	1
Feed pea	Antibody	1
Potato	Spider silk	1
Potato	Several pharmaceutical and technical traits	2
Potato	Non-plant carbohydrates	1
Potato	Spider silk-elastin fusion protein	1
Tobacco	Human alpha-1 anti-trypsin	1
Tobacco	Dog gastric lipase	7
Tobacco	Rabies virus G glycoprotein	1
Tobacco	Technical enzymes	1
Tobacco	Human collagen	3
Tobacco	Human glucocerebrosidase protein	1
Tobacco	Taxane diterpenoid	1

(Source: Joint Research Centre of the European Commission, 2009: http://gmoinfo.jrc.it/).

tions have to be granted at the EU level, involving all Member States and the European Commis-sion in decision-making. For activities involving GMOs conducted under strict containment (e.g. greenhouses or laboratories) Directive 2009/41/EC (EC, 2009) applies. Contained production is under the regulatory oversight of each particular Member State. When the end product of a molecular farming activity is a pharmaceutical, the safety or toxicological aspects of the product and its uses are covered by Regulation (EC) 726/2004 on medicinal products (EC, 2004).

A fundamental and internationally recognized principle of all regulations is that the use of a GMO is conditioned to a prior authorization delivered on a case-by-case basis after an in depth risk assessment of the activity has been performed. The objective of the risk assessment is to identify and evaluate potential adverse effects of the GMO on the receiving environment and on human health. The methodology and principles of the risk as-sessment are described for example in the Commission Decision 2002/623/EC (EC, 2002) for the EU and in the Cartagena Protocol on Biosafety at the international level (http://www.cbd.int/biosafety/protocol.shtml).

Although some authors have questioned whether the current GMO regulatory framework, particularly in the EU,

would be appropriate to deal with PMF (Spök, 2007), the main tendency is to consider that the risk assessment methodology and principles developed for the first generation of GM crops should be robust enough to evaluate risks from most applications of PMF. Besides, in the practice, the health and environmental risk assess-ments conducted for GM plants intended for molecular farming are currently performed in most countries according to the same criteria and procedures that are used for other GM plants (Peterson and Arntzen, 2004; Sparrow and Twyman, 2009; Spök et al., 2008). Never-theless, there are obviously key challenges from regulatory and risk assessment perspectives linked to the evaluation of PMF which deserve special attention and could justify the need for specific guidance, management measures and/or political choices. In this context, several national and international regulatory bodies have taken the option to develop guidelines, standards and procedures focusing on the use of these GM plants.

The US authorities have issued specific guidance to cover the risks associated with PMF (FDA, 2002; USDA, 2008). These documents provide information that an applicant should consider for addressing containment (to a facility such as a laboratory or greenhouse or during movement), confinement (to the field test site), and

environmental issues.

The Canadian Food Inspection Agency has also developed several rules, terms and conditions prescribed by new guidelines to address the additional environmental and human health concerns associated with the use of GM plants for molecular farming (CFIA, 2004b).

In the European Union, the European Food Safety Authority (EFSA) published recently a guidance document addressing specifically the risk assessment of GM plants used for non-food or non-feed purposes (EFSA, 2009). This guidance supplements a more general guidance for the risk assessment of genetically modified plants and derived food and feed (EFSA, 2006). EFSA only deals with the risks linked to the environmental release of the GM plant. The safety of the plant's product is considered by the European Medicines Agency, which has prepared guidelines on the safety and quality requirements of plant-derived drugs (EMEA, 2008).

Discussions on this topic are also going on at international level, notably in the framework of the Cartagena Protocol on Biosafety (see http://www.cbd.int/biosafety/default.shtml). An expert group has been established to consider the state of knowledge on risk assessment associated with future applications of modern biotechnology - specifically to organisms that are well advanced in the research and development stage and could enter the international system in the near future. This work intends also to address plants modified to produce pharmaceuticals.

BIOSAFETY ISSUES IN PLANT MOLECULAR FARMING

In the case of molecular farming plants, the focus of the evaluation for human, animal and environmental safety is on the risks resulting from accidental exposure to the GM plants by humans and animals since these plants are not intended for food or feed use nor for intentional environmental purposes.

In terms of environmental impacts, potential risks concern mainly, as for first-generation GM crops, the vertical transfer of genes from GM plants to non transgenic populations of the same or related species and the possible negative effects on mammals, birds, insects or microorganisms interacting directly or indirectly with crops.

Another key challenge linked to the biosafety eva-luation of plants that produce pharmaceutical/industrial compounds relates to the fact that these production systems might involve the cultivation in the open environment of GM plants that could inadvertently enter the feed or food chain via admixture, exposing animals or consumers to potentially toxic compounds. Unlike first-generation GM plants, some molecular farming plants are used specifically to produce substances that have an effect on humans or on higher animal species. They are also developed to produce large amount of active sub-stance, many times that produced in previous GM plants.

Many of these substances are biologically active and may have effects at low concentrations. As pointed out by some authors, virtually every pharmaceutical product currently on the market can cause allergic reactions in some people (Goldstein and Thomas, 2004). As a result, small amounts of pharmaceutical or indus-trial products may harm people that would inadvertently consume them. It should be noted however that oral exposure to these products is expected to be infrequent and of relatively short duration. In addition, most of the plant-made pharmaceutical proteins currently in the pipeline are not anticipated to have any pharmacological activity when ingested.

Recent reviews on safety issues associated with PMF and effective mechanisms to limit the risks include Commandeur et al. (2003), Liénard et al. (2007), Lu (2003), Mascia and Flavell (2004), Elbehri (2005), Murphy (2007), Sparrow et al. (2007), Sparrow and Twyman (2009) and Wolt et al. (2007).

CHOICE OF THE PRODUCTION PLATFORM

There are many factors to take into consideration when choosing the plant species that will be used as the host for producing the recombinant protein. On the one hand, the production strategy needs to comply with technical factors such as the required level of expression, the ways the product must be delivered or the quality of the end product (see e.g. Vancanneyt et al., 2009). On the other hand, from the biosafety viewpoint, the production host should be chosen taking into account the potential for and impact of exposure of the environment or of the food and feed chains. Consideration should be given to the potential impact of all aspects of the manufacturing process, including cultivation, harvest, transport, processing, purification, packaging, storage and disposal.

It is unlikely that any single plant species will satisfy all of the criteria required. Moreover, the best choice of host from a production perspective may not be the best from a biosafety point of view. Table 2 summarizes some of the main potential advantages and disadvantages for the four main host categories.

Using food plants as production hosts

The use of food crops as production systems for pharma-ceuticals or industrial compounds is a controversial issue. There are several arguments in favor of using food crops for PMF (Hennegan et al., 2005; Sparrow et al., 2007; Streatfield et al., 2003) and all the biopharmed products currently on the market are produced via maize production platforms.

However, many people are concerned about the risks such GM crops would pose in case they would inadver-tently enter the food or feed chain. A classic example of such accidental contamination is the ProdiGene incident in 2002. Following a standard crop rotation practice,

Table 2. Overview of host systems for PMF.

	Food plants	Non-food plants	Non-cultivated plants	Plant cells in culture
Examples	Maize, rice, potato, soybean, oilseed rape, banana, tomato	Tobacco, false flax	Duckweed, mosses, arabidopsis, algae	Tomato, tobacco, carrot
Main advantages	- Good knowledge of cultivation practices - In most cases, efficient transformation procedures - More options for the targeting and delivery of the recombinant protein	- Not part of the food chain - In some cases, good knowledge of cultivation practices and transformation procedures	- Not part of the food chain - Some of these plants can easily be grown in containment	- Propagated under containment - Easier maintenance of quality standards
Main disadvantages	- Risk of GM plants entering the food chain - Risk of gene transfer to related crop species - Co-existence aspects	- In some cases, production of toxins that could interfere with the processing of the pharmaceutical/ industrial compound	- In some cases, less knowledge about the genetics and biology of the plant - Very often, little experience with cultivation in the field	- Technical drawbacks (scaling-up ...) - Higher costs to maintain plant cells in culture

farmers had planted conventional soybeans for human consumption on land previously used to test in the field ProdiGene's GM maize that produces trypsin, a pancreatic serine protease. As a result, maize seed left from the transgenic crop grew into the soybean fields. Accidental contamination of the food chain does not only relate to safety aspects but creates also the potential for new questions about the financial liability to farmers and agricultural and food production industries. In this ProdiGene's case, the company accepted a civil penalty of US$ 250,000 and also covered the cost of destroying the soybean crop and the clean up steps that followed (Fox, 2003).

The policy currently adopted in the US foresees a strict requirement that plants grown for pharmaceutical or industrial compounds (and not approved for food and feed use) must stay clear of the food system under a zero-tolerance standard (USDA, 2006a). This will be possible only if a combination of strong physical and biological containment methods and monitoring measures is applied. If zero-tolerance is not set up as standard, several strategies could be envisaged to minimize the risks for human health associated with inadvertent admixture with food products.

A first option would be to have, for molecular farming plants grown in food crops, mandatory pre-marketing approval of the crops in question and related products under the food and feed regulation. Accordingly, a thorough food-safety analysis should be conducted to ensure that human exposure to the transgenic crop in the food supply would not result in any health risks. Such an approach would be similar to what currently exists in the EU as regards GMOs to be used as feed. According to Regulation EC 1829/2003 and to avoid experiences such as with Starlink maize in the US (Harl et al., 2003), these products can only be marketed if they are authorized both for feed and food uses.

Another option would be the adoption of threshold limits in cases of adventitious or technically unavoidable presence of molecular farming products in non-GM products at low enough levels that risks are minimal (Moschini, 2006; Spök, 2007). A threshold limit of 0.9% is currently applied at EU level for food and feed agricultural products. One can wonder however whether such an option would be acceptable to the food industry in the case of PMF, given the cost and the potential for a negative consumer reaction (USDA, 2002; Becker and Vogt, 2005).

Allowing the use of food crops for molecular farming is rather a matter of political choice. And there are many voices, including in the food industry, to stress that rather than attempting to impose ever more elaborate restrictions on the growing of food crops engineered for pharmaceutical and industrial purposes, it would be better to ban such applications altogether and to look for alternatives (Murphy, 2007; Union of Concerned Scientists, 2006).

Using non-food or non-cultivated plants

As mentioned in Table 2, the use of non-food or non-cultivated plants for PMF will in some cases be difficult and might pose new challenges in the risk assessment due to the lack of knowledge about the genetics and biology, the lack of domestication and/or the limited experience with the cultivation for some species (Murphy, 2007; Sparrow et al., 2007). Despite these drawbacks, it seems obvious that the adoption of non-food or feed plants will provide containment advantages and will largely reduce the possibility of unintentional contact and contgamination of the food or feed chains.

Among this category, tobacco is a very efficient production system in which a wide range of pharmaceutical products is currently being tested for production (Dunwell

and Ford, 2005). One example is the production by the company Planet Biotechnology of CaroRx™, a tobacco-made antibody to control dental caries (Wycoff, 2005).

Non-crop plants are also tested as production platform for pharmaceutical or industrial products. This includes the monocot *Lemna* (duckweed) (Cox et al., 2006), *Arabidopsis*, microalgae (Franklin and Mayfield, 2005; Walker et al., 2005) or mosses (Decker and Reski, 2007). All these species offer the advantage of a rapid reproductive rate and the possibility to be grown easily in contained facilities which offers many advantages in terms of biosafety.

Canadian authorities are explicitly recommending the use of non-food or feed crop species for PMPs (CFIA, 2004a). In the US, as mentioned above, some organizations are requesting to avoid the use of food crops to express pharmaceutical/industrial products. According to APHIS data, maize has dominated as the crop of choice until 2003 but, during the last years, there has been some drop in maize trials as a result of a move toward non-food crops for pharmaceutical trials. In the EU, plants for PMF are still considered on a case-by-case basis and no specific recommendations concerning the choice of the host for the production platform have been issued so far.

Cell cultures of transgenic plants

The safety of PMF can even be made greater by producing the recombinant product in cell cultures of transgenic plants (Hellwig et al., 2004; Murphy, 2007, Plasson et al., 2009). The main advantage is that suspension-cultured are made in bioreactors, a closed production system, avoiding problems associated with gene flow in the environment and potential contamination of the food and feed chains. In some cases, containment provides not only improved safety but allows also for easier recovery and purification of the molecule that is being produced. For instance, the recombinant protein can be fused with a signal sequence and secreted into the culture medium. In 2007, the company Protalix started clinical studies for the treatment of Gaucher's disease using recombinant human glucocerebrosidase produced in a carrot cell suspension culture (Shaaltiel et al., 2007).

Despite the fact that the adoption of cell culture technology has made progress during the last years, this strategy remains limited for the time being to a small number of well-characterized plant cell lines (such as tobacco, rice or *Arabidopsis*) and still needs improvements before it can be used in routine on a commercial scale.

COMPLEMENTARY STRATEGIES TO LIMIT THE POTENTIAL RISKS OF PMF

In addition to the choice of the most appropriate production platform for biopharming, a wide variety of complementary options involving either physical or biological methods are available to limit food/feed chains contamination or environmental impact of PMF. These options should be considered on a case-by-case basis, taking into account that they present different levels of reliability, that many of them are not mutually exclusive and that probably none of them will be able to achieve full protection of the environment or a zero level contamination of the food/feed chains. Amongst the most effective strategies are the physical containment of plants or cell cultures, the spatial containment of the GM plants, the development of biological confinement systems, the targeted expression of transgenes, and the development of inducible or transient expression systems.

Physical containment

Growing GM plants in physical structures is a first example of general preventive measure that would help avoiding contamination of the environment or of the food/feed chains. Potato, tobacco and other leafy crops such as alfalfa, lettuce and spinach are examples of plants that can be grown in contained facilities.

Various forms of physical containment can be envisaged: plastic tunnels; greenhouse production facilities (such as those used by the company Medicago to grow biopharmaceutical alfalfa for therapeutic proteins – (Zavon and Flinn, 2003)); laboratories or growth facilities such as phytotrons. Large-scale underground facilities (such as mines) have also been used in the US and Canada (Tackaberry et al., 2003).

Systematic restriction of PMF activities to contained facilities is a drastic solution that has been proposed recently in the US. A bill, which passed the Oregon Senate in 2005 but did not reach a final vote, asked for a four-year moratorium on PMF grown outdoors or using a food/feed crop, being for research or commercial purpose (http://www.oregonpsr.org/programs/campaignSafeFood.html).

Physical containment has however some limitations such as additional financial resources required to grow plants under containment or limitations of the scale of physical containment strategies (addressed in part by the development of large underground facilities in the US and Canada). When large quantities of pharmaceutical or industrial products are required or the crops do not grow well in isolated systems, open-field production remains necessary.

Spatial containment

Spatial separation includes several strategies aiming mainly at minimizing cross-fertilization between pharma/industrial crops and other crops. They could be of interest in particular in cases where the cultivation of pharma/industrial GM plants can be performed on small area.

Dedicated land

This approach consists in cultivating molecular farming

crops in regions where similar non-farming crops are not grown or in locations that are far removed from areas where non-farming crops are grown (FDA, 2002). This could virtually eliminate the risk of gene flow to non-farming plants and even of contamination of the food or feed chains. Although this approach may be difficult to set up and to monitor in practice, conceptual production model have been proposed to demonstrate the economic, safety, and environmental advantages of using dedicated growing area for transgenic nonfood products without switching between food and non-food uses on the same site (Howard and Hood, 2007).

Restricted use

In case the use of fully-dedicated land is not possible, field-testing or commercial production of PMPs and PMIs could be restricted in certain area for a defined number of growing seasons (depending on the plant) before the field can be used in the production of crops intended for use as food or feed. In that respect, US proposed rules for the regulation of field-testing of plants designed to produce pharmaceutical and industrial compounds include a prohibition against the planting of a food crop the year after the land was used for biopharming (Jones, 2003). If different molecular farming crops are to be grown on the same land in subsequent years, appropriate quality assurance measures should be adopted to control the quality of the raw agricultural material and the product.

Buffer and border zones

When pharmaceutical or industrial crops must be grown in the vicinity of food and feed crops, the potential of gene flow to nearby fields can be addressed using two different approaches that are already common practice in the management of GM plants of the first-generation.

Firstly, minimum isolation distances (buffer zones) can be imposed between fields of pharma/industrial crops and fields of the same species intended for food, feed or seed. Isolation distances will vary depending on the biology of the plant (self-pollinating, wind-borne,..). Secondly, it is also possible to plant a border of non-transgenic "trap" plants around the production field. These trap plants capture the majority of the pollen that might be produced by the GM plants. This method was originally developed to keep different conventional varieties from crossing with each other, but it can be applied as well for keeping pharma/industrial crops and other crops separate. While potentially reducing contamination via pollen dispersal, these approaches, used alone, will never achieve zero contamination.

Biological confinement

Reducing or preventing the flow of transgenes from the production site could also be achieved through biological

confinement, a strategy emphasized by the National Research Council in the US (NRC, 2004). Confinement strategies may be based on many different biological principles (Dunwell and Ford, 2005). In Europe, the Transcontainer project, funded from the European Commission Sixth Framework Programme, is working on new strategies to develop efficient and stable biological confinement systems for GM plants (http://www.transcontainer.org/UK/).

Plastid transformation

This confinement strategy consists in inserting the transgenes into the plant chloroplast genome instead of the plant nuclear genome. In addition to offering high rates of transgene expression and protein accumulation (Staub et al., 2000; De Cosa et al., 2001; Molina et al., 2004), molecular farming in chloroplasts has potential advantages at biosafety level (Bock, 2007; Ruf et al., 2007; Verma and Daniell, 2007). These advantages include the ability to control the site of gene insertion more precisely, and in many angiosperm species, the lack of transmission of transgenes via pollen due to the fact that plastid genes are maternally inherited. Since the pollen of many crop species does not contain chloroplasts, the transgene may not be transferable, conferring to this method a form of natural genetic confinement (Hagemann, 2004). Tobacco remains to date the species most amenable to plastid transformation although chloroplast genetic engineering has also been achieved successfully in other species, including tomato, cotton and potato, notably for the production of vaccine antigens and pharmaceutical proteins (human serum albumin, interferon...) (Daniell et al., 2001b,c, 2006). This strategy has also been considered as a recommended approach for gene confinement in a report of the Euro-pean Environment Agent(Eastham and Sweet, 2002).

Nevertheless the suitability of chloroplast genetic engineering for transgene containment remains to be assessed on a case-by-case basis. Plastid transgenesis is not necessarily a 100% reliable containment strategy. The transgene could escape from the chloroplast and enter the nucleus where it could under high selection pressure become active even if this would necessitate complex changes (Grevich and Daniell, 2005). Some studies have also shown the possibility of rare paternal plastid transmission (Svab and Maliga, 2007; Wang et al., 2004).

Male sterility

There are a large number of naturally occurring mechanisms of inducing male sterility. These mechanisms are very well known to the plant breeders and exploited to control hybridization in plants. In addition, male sterility can also be engineered in crops through recombinant nucleic acid technology, providing means by which trans-

genes are prevented from transferring to other plants. One of the most commonly used recombinant systems is the Barstar Barnase GM rapeseed developed in the 1990s by the company Plant Genetic Systems (PGS, Ghent, Belgium). In this system, promoter-directed expression of the destructive ribonuclease barnase from *Bacillus amyloquefaciens* inhibits pollen formation and results in male sterility of the transformed plants. The aim of the PGS system was to simplify the breeding of high-yield hybrid varieties. In principle it can also be used to biologically contain genetically modified plants and so prevent the spread of foreign genes. Male-sterile maize, oilseed rape, tobacco, sugar beet, sunflowers, potatoes, tomatoes, wheat, rice and cauliflower have already been produced as a result of this technology.

Alternative systems for inducing male sterility are also under development, e.g. by engineering cytoplasmic male-sterility (Dunwell and Ford, 2005; Ruiz and Daniell, 2005; Chase, 2006). Another approach to produce pollen-sterile plants is to target and kill off the cells that are involved in the development of the male flower (Brunner and Nilsson, 2004). Despite this great variety of methods and approaches to induce male sterility in plants there are few systems other than the Barstar Barnase that have been extensively tested in the field for their efficacy.

Gene Use Restriction Technologies (GURTs)

GURTs is a collective term gathering biotechnology-based switch mechanisms to restrict the use of genetic material. Two types of GURTs can be distinguished: variety use restriction (V-GURTs), in which sterile seeds are produced in the subsequent generation; and use restriction of a specific trait (T-GURTs), in which activation of a trait's expression is switched on or off through the external application of inducers. Seed sterility V-GURT is also well known as the so-called "Terminator" technology, a system developed under a cooperative research and development agreement between USDA and Delta and Pine Land Company (Oliver et al., 1998). This system has been much criticized in the public opinion as a mean for multinational seed companies to restrict the freedom of farmer of saving and re-sowing seeds. As a result such systems have been withdrawn from commercial development and even from field trials since several years. Despite of this, some plant developers would like to re-use the technology for the environmental containment of transgenic seed (V-GURTs) or transgenes (T-GURTs). However, to what extent these GURTs are in fact suitable for biological confinement is not clear due to the lack of scientifically based, reliable information on the practicability and reliability of these approaches (Lee and Natesan, 2006).

Other biological confinement strategies

Several other confinement strategies (sometimes exploi-

ting natural mechanisms) may also prove useful to reduce or eliminate gene flow among crops. They include apomixis (production of seed or fruit without the need for fertilization and pollination), cleistogamy (self-pollination and fertilization before flower opening), genomic incompatibility (placing the transgene on a genome of a polyploid plant species that is not compatible with related wild species), temporal and tissue-specific control via inducible promoters (see below) or transgenic mitigation (inclusion of transgenes such as genes inducing dwarfism or controlling seed dormancy, that compromise fitness in the hybrid under non-agricultural conditions) (Daniell, 2002).

It is important to realize that most of the biological confinement mechanisms described above are far from being used for commercial production (Ellstrand, 2003). In addition, some of them (such as chloroplast transformation or male sterility) do not prevent gene transfer resulting from seed dispersal during cultivation, harvest or transport. It is therefore unlikely that biological measures will totally prevent gene flow. To achieve a high level of protection of the environment, the choice to apply one or several of these technologies might be done on a case-by case basis, in parallel or in combination with other containment measures and depending on the intrinsic characteristics of each specific crop.

Targeted expression

Targeting the expression of the product of interest to a few specific plant parts or subcellular compartments represents another strategy to reduce the unintended exposure to a pharmaceutical or industrial product. This can be achieved by the use of tissue specific promoters. There are several options available which can be implemented depending on the potential advantages and disadvantages in terms of increased yields, simplification of the purification process and biosafety aspects.

Expression from or in roots

The engineering of plants to secrete molecules from their roots into medium is a technology that is currently being tested for the production of recombinant proteins in molecular farming (Vitale and Pedrazzini, 2005). This technology has the advantage of allowing the production of proteins in a bioreactor (thus in contained facilities) but is still far from being used at the commercial level. On the opposite, another strategy that is currently under investigation consists in blocking release or diffusion of the product from the roots of transgenic plants for retention in the endoplasmic reticulum. This would contribute to the environmental safety of crops producing PMPs by reducing the potential release into the environment of toxins or other pharmaceutical products that could cause "protein pollution" and represent a hazard for soil and rhizosphere microbial communities (Pizzuti and Daroda,

2008).

Expression in edible parts

If the product is to be consumed then production in a fruit or edible portion of a plant is a possible choice. A well-known example is the use of plants such as banana, potato, lettuce or carrot as potential vehicles for a vaccine against hepatitis B (Kumar et al., 2007). It should be noted however that the idea of using fruit or vegetables directly as vaccine is now being reconsidered seriously due to the problems associated with adequate dosage of the recombinant product and the potential for inadvertent mixing with material destined for food or feed chain.

Expression in seeds

Another approach is the production of the pharmaceutical or industrial product specifically in seeds from cereals, grain legumes or oilseeds. There are several practical advantages to production in seeds that include accumulation of proteins in a stable environment in which they are protected from degradation, convenience of harvest, easy transportation from farm to production factories, facilitated downstream protein extraction and convenience of seeds for end use if the product is to be consumed directly without purification (Stoger et al., 2005). On the other hand, production in seeds (like in fruits) does not address adequately the issue of pollen transfer since the transgenic plants must go through a flowering cycle.

Expression in other parts of the plant

Recombinant proteins can also be targeted into specific plant organs that can be easily harvested and removed from the field such as leaves or stems. For example, materials produced in the vegetative parts of alfalfa or tobacco can be harvested prior to flower and pollen development. In the case of maize, the tassels could be manually removed. Although in these cases the plants do not need to flower, these approaches raise other biosafety issues such as the potential exposure of herbivores to recombinant proteins expressed in the leaves (Sparrow et al., 2007).

Temporal confinement

Temporal confinement can occur through physical or biological methods.

Use of "a typical" growing seasons

This strategy of physical temporal confinement consists in planting molecular farming crops at different times than food and feed crops to prevent an overlap in flowering times, therefore decreasing the potential for pollen transfer. This option is however difficult to implement in practice because of the difficulty to control environmental factors influencing the timing of flowering in plants.

Post-harvest inducible expression

In this biological approach, the transgene is not expressed at all in the plants in the field but the molecular farming product will only be formed when the harvested plant material is removed to a processing facility and exposed to a chemical or environmental stimulus that activates expression of the transgene (Cramer et al., 1999). This can be achieved for example with the use of an inducible promoter or through the expression of the product in a form that must be treated for activation (e.g. hirudin is produced as a fusion protein and is inactive in this form; it is activated only after it is purified from seeds). Inducible expression of the GM trait requires of course stringent application conditions. In addition, such system may prove useful only in very limited cases where the protein of interest may not require extended or constant expression throughout the life cycle of a transgenic plant.

Transient expression

This approach is being used more and more extensively by companies aiming to the fast production of vaccines or other products on a small or medium scale (Streatfield and Howard, 2003; Vézina et al., 2009). High levels of protein expression can be achieved for a short time. In this case, the transgene is only present temporarily in the plant cells and cannot be inherited by the next generation. Another safety advantage is that the technique is generally applied in a contained environment. Different DNA or RNA delivery approaches can be used to generate transient expression: direct delivery methods (e.g. particle bombardment or microinjection), viral vector systems (Gils et al., 2005) or bacterial infection systems. One of the most promising transient expression systems is agroinfiltration which is based on the inoculation of leaves with recombinant *Agrobacterium tumefaciens* (D'Aoust et al., 2008, 2009; Marillonnet et al., 2005).

ADDITIONAL CONSIDERATIONS

Handling, transport, equipment and personnel

Under normal agricultural and grain-handling channels, equipment is not entirely cleaned out and co-mixture of different seeds is therefore always possible. The use of dedicated equipment for planting, harvesting and transporting molecular farming plants and even of dedicated facilities for the processing of source plant materials provides an additional protection against accidental mixing with plants entering the food/feed supply. Ideally,

containers used to transport and/or store harvested products should also be dedicated to PMF activities, although this will be very difficult to achieve in practice for the larger transportation equipment (such as ships). A further option to improve safety is the clear labeling of containers of harvested material, indicating that the material is not to be used for food or feed purposes. All these options would certainly greatly contribute to limit the potential contamination of the food and feed chains but their implementation could be refrained in particular due to the additional costs that dedicated equipment and facilities could represent for the producers.

To avoid or limit co-mixture in the case where food crops are used as production systems for pharmaceuticals or industrial compounds, a comprehensive management system should be considered including the development and implementation of appropriate procedures (Mascia and Flavell, 2004). Such management system could be based on or complement those adopted in the framework of production protocols. These are designed to maintain product integrity and consistency and prevent contamination of the plant-made end-product during all stages of production. These procedures cover e.g. the cleaning of equipment and storage faci-lities, the harvesting of the source material, the control over the inventory and disposition of viable seeds...

In addition to the control of environmental exposure to plant-made pharmaceuticals or industrial products, it could be necessary to implement protective measures to limit exposure of workers during all phases of production, harvest and processing (occupational safety). Indeed, the production of PMPs or PMIs can result in direct exposures of workers participating in the production and processing of the product (Wolt et al., 2007). For instance, touching or inhaling of plant vaccine materials during production may lead to oral tolerance or allergenicity (Kirk et al., 2005). These concerns are in essence not different than those associated with any pharmaceutical manufacturing process where the routes of worker exposure may involve dermal contact, hand-to-mouth transfers, or inhalation. Protected measures will have to be implemented on a case by case basis (depending on the allergenic or toxic effects of the molecule) for both cultivating sites and processing units, taking into account that the place are in some cases particular in the case of PMF (field, greenhouse...).

Waste management

Residual material left on the cultivating site and in the storage facilities and by-products of processing could become an issue in particular if molecular farming activities increase in scale. Appropriate measures (consigned in standard operating procedures) should therefore be taken to handle the waste in order to ensure that the material will not enter the human or animal food chain or impact the environment.

The use of the remaining biomass has been proposed by some companies as one way to defray the high costs of purification. This approach has been however criticized for the additional level of risk it would pose to food safety (Freese, 2002). In any case, decisions concerning the use of left over materials as animal feed or derived products (such as starch produced from GM potato tubers) should be considered on a case by case basis depending on the nature or risk associated with the molecular farmed material and the proposed end use.

Post-market management measures

If the molecular farming plant is grown in the field, the monitoring of the production site, supported by an appropriate inspection plan, will be required as for any GM plant cultivated outdoor. According to European Directive 2001/18/EC, the objective of post-market monitoring is (i) to confirm that any assumptions regarding the occurrence and impact of potential adverse effects of the GMO or its use in the risk assessment are correct, and (ii) to identify the occurrence of adverse effects of the GMO or its use on human health or the environment which were not anticipated in the risk assessment. Any unusual occurrence should be reported to the appropriate regulatory authority.

Detection and quantification of GMOs are key elements of some post-market management system. This is the case in particular in the EU for GMOs used as food or feed (EC, 2003). As a consequence, adequate molecular methods must be available that enable the detection, identification and quantification of each GMO individually (the so-called "transformation event"). Such a system also aims at detecting GMOs that are not authorized in the relevant jurisdiction and therefore could pose a potential risk for consumers if they enter the food or feed chain. This would also be the case for molecular farming plants or parts thereof in case of accidental contamination, especially when a considerable number of diverse crops are reached.

In that respect, potential management tools can be envisaged to support molecular detection and quantification of the pharma plant accidentally appearing in food or feed. For instance, standard production protocols may involve tagging of such plants with a generic non-food/feed GM plant-specific DNA sequence identifier, apart from an event-specific marker, preferably being devoid of open reading frame (EFSA, 2009). This risk management tool would facilitate event-specific DNA detection of the GMO.

The introduction of morphological markers (visual confinement) is another strategy that could help in the identification and traceability of crops expressing pharmaceutical or industrial products. In this case, the bioengineered pharmaceutical or industrial plant line is made visually distinctive from its food or feed counterpart. This might include for example the use of genetic markers that alter the physical appearance of the plant (e.g. a novel color or leaf pattern), or change the condi-

tions under which a plant will grow (for example the use of auxotrophic marker genes) (Commandeur et al., 2003; FDA, 2002). Such measure would of course help when contamination has occurred but may not prevent it in the first place. In addition, this would imply adding new heterologous genes in the plant genome which could make the risk assessment of such GMOs even more complex.

CONCLUSION

Plants offer a wide range of technical options for the production of pharmaceutical or industrial products. Given the great diversity of potential production systems and target molecules, many strategies could be implemented to limit the potential negative impact on the environment and the inadvertent contamination of food or feed associated with PMF, including the choice of the production host, the implementation of physical and biological containment methods and the adoption of relevant management practices. Some of these strategies will involve relatively low-tech measures, such as meticulous planning and supervision of each step in the production process. Other might involve complex procedures and much additional cost. Any final decision in that respect should be based on the results of a case by case science-based risk assessment.

One of the key questions to be answered in the case of PMF is how far zero-tolerance contamination into the food and feed chains should be met. Lowering the contamination level to the zero-tolerance level is advocated by many (and this is currently the regulatory standard adopted in the US) due to several cases of unexpected contamination of the food chain by transgenic crops over the past decade. In practice, a 100% guarantee of zero contamination might most probably be achievable only by totally precluding field cultivation to ensure complete isolation from the food and feed chains. Physical containment could encompass all aspects of the development and production processes, from breeding and testing to commercial production.

Such an option could apply to certain type of molecular farming. But open field cultivation might be in other cases the only commercially acceptable option for the production of some PMP or PMI (Spök and Karner, 2008). To limit the possibility of inadvertent entry into the food chain, the use of non-food crops such as tobacco might be envisaged. Nevertheless, as discussed earlier, using food crops for the production of PMPs or PMIs has also some merits that should be considered. Therefore, it is maybe not relevant to exclude *a priori* major crop plants as hosts, particularly if the target molecule poses little or no risk to environmental or human health.

Field production of PMPs or PMIs has at present be limited to trials covering only a relatively few hectares globally. Different pictures emerge from speculations about the acreage required for overall PMP/PMI production. For some authors the area needed by 2014 is estimated not to exceed 10,000 ha globally and should not have a large direct impact on agriculture (Graff and Moschini, 2004; Wolt et al., 2007). According to other estimations some PMF applications could require much larger acreage (Spök and Karner, 2008). It seems evident that a simultaneous cultivation of various types of pharmaceutical or industrial plants might challenge the coexistence regimes currently established for GM and non-GM agriculture (particularly in the EU), rendering segregation measures much more complex and costly to implement than with the first generation of GM products.

In any case, open field cultivation will always need additional stringent measures. Spatial containment strategies have been presented as a mean to minimize cross-fertilization between pharma/industrial crops and other conventional agricultural crops. Targeted expression of the recombinant product to specific plant parts or compartments represents another way of reducing the unintended exposure to a pharmaceutical or industrial product. Last but not least, several methods of biological containment have been reviewed and discussed in this paper. They will certainly add an extra layer of isolation and should be of considerable help in reducing the possibility of contamination of food or feed. Unfortunately, most of these strategies are still in the development phase and it will most probably still make some time before they provide effective means to create reliable biological containment systems.

Plant molecular farming opens the door for the production of pharmaceuticals and industrial compounds at low costs and with several potential advantages com-pared to microbial or mammalian production platforms. However, any developments in this field must be framed by a thorough evaluation of the risks to the food/feed supply system and the environment. This assessment must take into account the characteristics of the host plant, the product, the intended production area, and relevant handling practices. Although there is a general agreement that the health and environmental risk assessments conducted for GM plants intended for molecular farming can be performed according to the same criteria and procedures that are used for other GM plants, many countries have also acknowledged the need to develop specific guidance focusing on PMF. This in turn shows the need for a strong and adaptable regulatory framework to support the specifics of plant molecular farming.

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REFERENCES

Becker G, Vogt D (2005). Regulation of Plant-Based Pharmaceuticals. CRS Report for Congress. Order Code RS21418, March 8.

Bock R (2007). Plastid biotechnology: prospects for herbicide and insect resistance, metabolic engineering and molecular farming. Curr. Opin. Biotechnol. 18: 100-106.

Brunner AM, Nilsson O (2004). Revisiting tree maturation and floral initia-tion in the poplar functional genomics era. New Phytologist 164: 43-51.

CFIA (2004a). Directive Dir2000-07: Conducting Confined Research Field Trials of Plant with Novel Traits in Canada. http://www.inspection.gc.ca/english/plaveg/bio/dir/dir0007e.shtml [accessed 25 August 2009].

CFIA (2004b). Directive 94-08. Assessment Criteria for Determining Environmental Safety of Plants with Novel Traits. http://www.inspection.gc.ca/english/plaveg/bio/dir/dir9408e.shtml [accessed 25 August 2009].

Chase CD (2006). Genetically engineered cytoplasmic male sterility. Trends Plant Sci. 11(1):7-9.

Commandeur U, Twyman RM, Fischer R. (2003). The biosafety of molecular farming in plants. Ag. Biotech. Net 5: 1-9.

Cox KM, Sterling JD, Regan JT, Gasdaska JR, Frantz KK, Peele CG, Black A, Passmore D, Moldovan-Loomis C, Srinivasan M, Cuison S, Cardarelli PM, Dickey LF (2006). Glycan optimization of a human monoclonal antibody in the aquatic plant Lemna minor. Nat. Biotechnol. 24: 1591–1597.

Cramer C, Boothe JG, Oishi KK (1999). Transgenic plants for therapeutic proteins: linking upstream and downstream technologies. Curr. Trop. Microbiol. Immunol. 240: 95-118.

D'Aoust MA, Lavoie PO, Couture MM, Trépanier S, Guay JM, Dargis M, Mongrand S, Landry N, Ward BJ, Vézina LP (2008). Influenza virus-like particles produced by transient expression in Nicotiana benthamiana induce a protective immune response against a lethal viral challenge in mice. Plant Biotechnol. J. 6: 930-40.

D'Aoust MA, Lavoie PO, Belles-Isles J, Bechtold N, Martel M, Vézina LP. (2009). Transient expression of antibodies in plants using syringe agro- infiltration. Methods Mol. Biol. 483: 41-50.

Daniell H, Streatfield SJ, Wycoff K. (2001a). Medical molecular farming: production of antibodies, biopharmaceuticals and edible vaccines in plants. Trends Plant Sci. 6: 219-226.

Daniell H, Khan MS, Allison L (2001b). Milestones in chloroplast genetic engineering: an environmental friendly era in biotechnology. Trends Plant Sci. 7: 84-91.

Daniell H, Lee SB, Panchal T, Wiebe PO (2001c). Expression and assembly of the native cholera toxin B subunit gene as functional oligo-mers in transgenic tobacco chloroplasts. J. Mol. Biol. 311: 1001-1009.

Daniell H (2002). Molecular strategies for gene containment in transgenic corps. Nat. Biotechnol. 20: 581-586.

Daniell H (2006). Production of biopharmaceuticals and vaccines in plants via the chloroplast genome. Biotechnol. J. 1(10): 1071-1079.

De Cosa B, Moar W, Lee SB, Miller M, Daniell H (2001). Over-expression of the Bt cry2Aa2 operon in chloroplasts leads to formation of insecticidal crystals. Nat. Biotechnol. 19: 71-74.

Decker EL, Reski R (2007). Moss bioreactors producing improved biophar-maceuticals. Curr. Opin. Biotechnol. 18: 393-398.

Dunwell JM, Ford CS (2005). Technologies for biological containment of GM and non-GM crops. Defra contract CPEC 47. Final report. http://www.gmo-safety.eu/pdf/biosafenet/Defra_2005.pdf [accessed 25 August 2009].

Eastham K, Sweet J (2002). Genetically Modified Organisms (GMOs): The Significance of Gene Flow Through Pollen Transfer. Environmental Issue Report 28 (European Environment Agency, Copenhagen, Denmark).

EC (2001). Council Directive 2001/18/EC of 12 March 2001 on the deliberate release into the environment of genetically modified organisms and repealing Council Directive 90/220/EEC. Official J. Eur. Union L106:1-38.

EC (2002). Commission Decision 2002/623/EC of 24 July establishing guidance notes supplementing Annex II to Directive 2001/18/EC of the European Parliament and of the Council on the deliberate release into the environment of genetically modified organisms and repealing Council Directive 90/220/EEC. Official J. Eur. Union L200: 22-33.

EC (2003). Regulation (EC) No 1829/2003 of 22 September on genetically modified food. Official Journal of the European Union L268: 1-23.

EC (2004). Regulation (EC) No 726/2004 of the European Parliament and of the Council of 31 March 2004 laying down Community procedures for the authorisation and supervision of medicinal products for human and veterinary use and establishing a European Medicines Agency. Official J. Eur. Union L136:1-33.

EC (2009). Directive 2009/41/EC of the European Parliament and of the Council of 6 May on the contained use of genetically modified micro-organisms. Official J. Eur. Union L125: 75-97.

EFSA (2006). Guidance document of the scientific panel on genetically modified organisms for the risk assessment of genetically modified plants and derived food and feed. EFSA J. 99: 1-100.

EFSA (2009). Scientific Opinion on Guidance for the risk assessment of genetically modified plants used for non-food or non-feed purposes. EFSA-Q-2007-176. EFSA J. 1164: 1-42.

EMEA (2008). Guidelines on the quality of biological active substances produced by stable transgene expression in higher plants. EMEA/CHMP/BWP/48316/2006.

Elbehri A (2005). Biopharming and the Food System: Examining the Potential Benefits and Risks. Ag. Biol. Forum 8(1): 18-25.

Ellstrand NC (2003). Going to 'great lengths' to prevent the escape of genes that produce specialty chemicals. Plant Physiol. 132: 1770-1774.

FDA (2002). Draft Guidance for Industry. Drugs, Biologics, and Medical Devices Derived from Bioengineered Plants for Use in Humans and Animals. http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatory Information/Guidances/ucm124811.pdf [accessed 25 August 2009].

Federal Register Notice (2003). 68 FR 11337-11340. http://www.aphis.usda.gov/brs/pdf/7cfr.pdf [accessed 25 August 2009].

Fischer R, Stoger E, Schillberg S, Christou P, Twyman RM (2004). Plant-based production of biopharmaceuticals. Curr. Opin. Plant Biol. 7: 152-158.

Floss DM, Falkenburg D, Conrad U (2007). Production of vaccines and therapeutic antibodies for veterinary applications in transgenic plants: An overview. Transgenic Res. 16: 315-332.

Fox JL (2003). Puzzling industry response to prodigene fiasco. Nat. Biotechnol. 21: 3-4.

Franklin SE, Mayfield SP (2005). Recent developments in the production of human therapeutic proteins in eukaryotic algae. Expert Opin. Biol. Ther. 5: 1225-235.

Freese B (2002). Manufacturing Drugs and Chemicals in Crops. Biopharming poses new threats to consumers, farmers, food companies and the environment. Genetically Engineered Food Alert. http://www.kahea.org/gmo/pdf/FOE_biopharm_exsumm.pdf [25 August 2009].

Gils M, Kandzia R, Marillonnet S, Klimyuk V, Gleba Y (2005). High-yield production of authentic human growth hormone using a plant virus-based expression system. Plant Biotechnol. J. 3:613-20.

Goldstein DA, Thomas JA (2004). Biopharmaceuticals Derived from Genetically Modified Plants. QJM: Int. J. Med. 97: 705-716.

Gomord V, Chamberlain P, Jefferis R, Faye L (2005). Biopharmaceutical production in plants: problems, solutions and opportunities. Trends Biotechnol. 23:559-65.

Graff G, Moschini G (2004). Pharmaceuticals and industrial products in crops: economic prospects and impact on agriculture. Iowa Ag. Rev. 10: 4–11.

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

Hagemann R (2004). The sexual inheritance of plant organelles. In: Mole-cular Biology and Biotechnology of Plant Organelles. Daniell H, Chase C (Ed.). Springer Publishers, Dordrecht, Netherlands pp. 93-113.

Harl NE, Ginder RG, Hurgurgh CR, Moline S (2003). The Starlink™ Situation. Ames, IA: Iowa State University Extension. http://www.biotech-info.net/0010star.PDF [accessed 25 August 2009].

Hellwig S, Drossard J, Twyman RM, Fischer R (2004). Plant cell cultures for the production of recombinant proteins. Nat. Biotechnol. 22:1415–1422.

Hennegan K, Yang DC, Nguyen D, Wu LY, Goding J, Huang JM, Guo FL, Huang N, Watkins S (2005). Improvement of human lysozyme

expression in transgenic rice grain by combining wheat (*Triticum aestivum*) puroindoline b and rice (*Oryza sativa*) Gt1 promoters and signal peptides. Transgenic Res. 14: 583-592.

Hood EE, Witcher DR, Maddock S, Meyer T, Baszczynski C, Bailey M, Flynn P, Register J, Marshall L, Bond D (1997). Commercial production of avidin from transgenic maize: characterization of transformant, pro-duction, processing, extraction and purification. Mol. Breed. 3: 291-306.

Hood EE (2002). From green plants to industrial enzymes. Enzyme Microbial Technol. 30: 279-283.

Howard JA (2005). Commercialization of Biopharmaceutical and Bioindustrial Proteins from Plants. Crop Sci. 45: 468-472.

Howard JA, Hood EE (2007). Methods for growing nonfood products in transgenic plants. Crop Sci. 47:1255-1262.

Jones PBC (2003). Cultivating Ground Rules for Biopharming. ISB News Report. http://www.isb.vt.edu/news/2003/news03.jun.html [accessed 25 August 2009].

Kirk DD, McIntosh K, Walmsley AM, Peterson RK (2005). Risk analysis for plant-made vaccines. Transgenic Res. 14: 449-162.

Kumar GBS, Ganapathi TR, Bapat VA (2007). Production of Hepatitis B Surface Antigen in Recombinant Plant Systems: An Update. Biotechnol. Progress 23: 532 - 539.

Liénard D, Sourrouille C, Gomord V, Faye L. (2007). Pharming and transgenic plants. Biotechnol Annu Rev. 13: 115-47.

Lee D, Natesan E (2006) Evaluating genetic containment strategies for transgenic plants. Trends Biotechnol. 24: 109-114.

Lu BR (2003). Transgene containment by molecular means - is it possible and cost effective? Environ. Biosafety Res. 2: 3–8.

Ma JK, Drak PM, Christou P (2003). The production of recombinant pharmaceutical proteins in plants. Nat. Rev. Genet. 4: 794-805.

Ma JK, Barros E, Bock R, Christou P, Dale PJ, Dix PJ, Fischer R, Irwin J, Mahoney R, Pezzotti M, Schillberg S, Sparrow P, Stoger E, Twyman RM (2005a). Molecular farming for new drugs and vaccines. Current perspectives on the production of pharmaceuticals in transgenic plants. EMBO Rep. 6: 593-599.

Ma JK, Chikwamba R, Sparrow P, Fischer R, Mahoney R, Twyman RM (2005b). Plant-derived pharmaceuticals – the road forward. Trends Plant Sci. 10: 580-585.

Marillonnet S, Thoeringer C, Kandzia R, Klimyuk V, Gleba, Y (2005). Systemic Agrobacterium tumefaciens-mediated transfection of viral replicons for efficient transient expression in plants. Nat. Biotechnol. 23: 718–723.

Mascia PN, Flavell RB (2004). Safe and acceptable strategies for producing foreign molecules in plants. Curr. Opin. Plant Biol. 7: 189-195.

Molina A, Hervas-Stubbs S, Daniell H, Mingo-Castel AM, Veramendi J (2004). High-yield expression of a viral peptide animal vaccine in transgenic tobacco chloroplasts. Plant Biotechnol. J. 2: 141-153.

Moschini G (2006). Pharmaceutical and Industrial Traits in Genetically Modified Crops: Co-existence with Conventional Agriculture. Working Paper 06-WP 429. Center for Agricultural and Rural Development.

Murphy DJ (2007). Improving containment strategies in biopharming. Plant Biotechnol. J. 5: 555-569.

NARA (2005). Introductions of plants genetically engineered to produce industrial compounds. Federal register (USA), 70 (85):23009-23011.

NRC (2004). Biological Confinement of Genetically Engineered Organisms. The National Academies Press, Washington DC, USA, ISBN 0-309-09085-7.

Oliver MJ, Quisenberry JE, Trolinder NLG, Keim DL (1998). Control of plant gene expression. US patent 5,723,765.

Peterson RKD, Arntzen CJ (2004). On risk and plant-based biopharmaceuticals. Trends Biotechnol. 22:64-66.

Pizzuti F, Daroda L (2008). Investigating recombinant protein exudation from roots of transgenic tobacco. Environ. Biosafety Res. 7: 219-26.

Plasson C, Michel R, Lienard D, Saint-Jore-Dupas C, Sourrouille C, de March GG, Gomord V (2009). Production of recombinant proteins in suspension-cultured plant cells. Methods Mol. Biol. 483: 145-161.

Raskin I, Ribnicky DM, Komarnytsky S, Ilic N, Poulev A, Borisjuk N, Brinker A, Moreno DA, Ripoll C, Yakoby N, O'Neal JM, Cornwell T, Pastor I, Fridlender B (2002). Plants and human health in the twenty-first century. Trends Biotechnol. 20: 522-531.

Ruf S, Karcher D, Bock R (2007). Determining the transgene containment level provided by chloroplast transformation. Proc. Natl. Acad. Sci. USA, 104(17): 6998-7002.

Ruiz ON, Daniell H (2005). Engineering cytoplasmic male sterility via the chloroplast genome by the expression of beta-ketothiolase. Plant Physiol. 138: 1232-1246.

Shaaltiel Y, Bartfeld D, Hashmueli S, Baum G, Brill-Almon E, Galili G, Dym O, Boldin-Adamsky SA, Silman I, Sussman JL, Futerman AH, Aviezer D (2007). Production of glucocerebrosidase with terminal mannose glycans for enzyme replacement therapy of Gaucher's disease using a plant cell system. Plant Biotechnol. J. 5: 579-90.

Sparrow PA, Irwin JA, Dale PJ, Twyman RM, Ma JK (2007). Pharma-Planta: road testing the developing regulatory guidelines for plant-made pharmaceuticals. Transgenic Res. 16(2): 147-61.

Sparrow PA, Twyman RM (2009). Biosafety, Risk Assessment and Regu-lation of Plant-made Pharmaceuticals. Methods Mol. Biol. 483:341-53.

Spök A (2007). Molecular farming on the rise - GMO regulators still walking a tightrope. Trends Biotechnol. 25: 74-82.

Spök A, Karner S (2008). Plant Molecular Farming, Opportunities and Challenges. JRC Technical Report EUR 23383 EN. Luxembourg: Office for Official Publications of the European Communities.

Spök A, Twyman RM, Fischer R, Ma JKC, Sparrow PAC (2008). Evolution of a regulatory framework for pharmaceuticals derived from genetically modified plants. Trends Biotechnol. 646:1-12.

Staub JM, Garcia B, Graves J, Hajdukiewicz PT, Hunter P, Nehra N, Paradkar V, Schlittler M, Carroll JA, Spatola L, Ward D, Ye G, Russell DA (2000). High-yield production of a human therapeutic protein in tobacco chloroplasts. Nat. Biotechnol. 18: 333–338.

Stoger E, Ma JK, Fischer R, Christou P (2005). Sowing the seeds of success: pharmaceutical proteins from plants. Curr. Opin. Biotechnol. 16(2): 167-73.

Streatfield SJ, Howard JA (2003). Plant-based vaccines. Int. J. Parasitol. 33: 479-493.

Streatfield SJ, Lane JR, Brooks CA, Barker DK, Poage ML, Mayor JM, Lamphear BJ, Drees CF, Jilka JM, Hood EE, Howard JA (2003). Corn as a production system for human and animal vaccines. Vaccine. 21: 812-815.

Svab Z, Maliga P (2007). Exceptional transmission of plastids and mito-chondria from the transplastomic pollen parent and its impact on transgene containment. Proc. Natl. Acad .Sci. U S A. 104: 7003-7008.

Tackaberry ES, Prior F, Bell M, Tocchi M, Porter S, Mehic J, Ganz PR, Sardana R, Altosaar I, Dudani A (2003). Increased yield of heterologous viral glycoprotein in the seeds of homozygous transgenic tobacco plants cultivated underground. Genome 46: 521-526.

Twyman R, Stoger E, Schillberg S, Christou P, Fischer R (2003). Molecular farming in plants: host systems and expression technology. Trends Biotechnol. 21: 570-578.

Twyman RM, Schillberg S, Fischer R (2005). The transgenic plant market in the pharmaceutical industry. Expert Opin. Emerg. Drugs 10: 185–218.

Union of Concerned Scientists (2006). UCS Position Paper: Pharmaceutical and Industrial Crops. UCS. http://www.ucsusa.org/food_and_environment/genetic_engineering/ucs-position-paper.html [accessed 25 August 2009].

USDA (2002). Pharming the field: A Look at the Benefits and Risks of Bioengineering Plants to Produce Pharmaceuticals. Proceedings from a workshop sponsored by the Pew Initiative on Food and Biotechnology, the U.S. Food and Drug Administration and the Cooperative State Research, Education and Extension Service of the U.S. Department of Agriculture.

USDA (2006a). Permitting genetically engineered plants that produce pharmaceutical compounds. BRS Factsheet.http://www.aphis.usda.gov/publications/biotechnology/content/printable_version/BRS_FS_pharmaceutical_02-06.pdf [accessed 25 August 2009].

USDA (2008). Guidance for APHIS Permits for Field Testing or Movement of Organisms Intended for Pharmaceutical or Industrial Use. http://www.aphis.usda.gov/brs/pdf/Pharma_Guidance.pdf [accessed on 25 August 2009].

Vancanneyt G, Dubald M, Schröder W, Peters J, Botterman J (2009). A case study for plant-made pharmaceuticals comparing different plant expression and production systems. Methods Mol. Biol. 483: 209-221.

Verma D, Daniell H (2007). Chloroplast vector systems for biotechnology applications. Plant Physiol. 145: 1129-1143.

Vézina LP, Faye L, Lerouge P, D'Aoust MA, Marquet-Blouin E, Burel C, Lavoie PO, Bardor M, Gomord V (2009). Transient co-expression for fast and high-yield production of antibodies with human-like N-glycans in plants. Plant Biotechnol. J. 7: 442-455.

Vitale A, Pedrazzini E (2005). Recombinant pharmaceuticals from plants: The plant endomembrane system as bioreactor. Mol. Interven. 5: 216-225.

Walker TL, Purton S, Becker DK, Collet C (2005). Microalgae as bioreactors. Plant Cell Rep 24: 629-641.

Wang T, Li Y, Shi Y, Reboud X, Darmency H, Gressel J (2004). Low frequency transmission of a plastid-encoded trait in Setaria italica. Theor. Appl. Genet. 108: 315-320.

Witcher DR (1998). Commercial production of b-glucuronidase (GUS): a model system for the production of proteins in plants. Mol. Breed. 4: 301-312.

Wolt JD, Karaman S, Wang K (2007). Risk assessment for plant-made pharmaceuticals. CAB Reviews: Perspectives in Agriculture, Veterinary Science, Nutr. Nat. Resour. 2(12): 1-9.

Woodard SL, Mayor JM, Bailey MR, Barker DK, Love RT, Lane JR, Delaney DE, McComas-Wagner JM, Mallubhotla HD, Hood EE, Dangott LJ, Tichy SE, Howard JA (2003). Maize (Zea mays)-derived bovine trypsin: characterization of the first large-scale, commercial product from transgenic plants. Biotechnol. Appl. Biochem. 38: 123-130.

Wycoff KL (2005). Secretory IgA antibodies from plants. Curr. Pharm. Des. 11: 2429-37.

Zavon JA, Flinn JE. (2003). Future of pharming involves look at big picture. Feedstuffs 75(25): A11.