Genetically modified plants for non-food or non-feed purposes: Straightforward screening for their appearance in food and feed

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Genetically modified (GM) plants aimed at producing food/feed are part of regular agriculture in many areas of the World. Commodity plants have also found application as bioreactors, designated non-food/non-feed GM (NFGM) plants, thereby making raw material for further refinement to industrial, diagnostic or pharmaceutical preparations. Many among them may pose health challenge to consumers or livestock animals, if occurring in food/feed. NFGM plants are typically released into the environment, but are grown under special oversight and any among several containment practices, none of which provide full protection against accidental dispersal. Adventitious admixture with food or feed can occur either through distributional mismanagement or as a consequence of gene flow to plant relatives. To facilitate NFGM surveillance we propose a new mandatory tagging of essentially all such plants, prior to cultivation or marketing in the European Union. The suggested tag – Plant-Made Industrial or Pharmaceutical Products Tag (PMIP-T) – is envisaged to occur as a transgenic silent DNA identifier in host plants and designed to enable technically simple identification and characterisation of any NFGM. Implementation of PMIP-T would permit inexpensive, reliable and high-throughput screening for NFGM specifically. The paper outlines key NFGM prospects and challenges as well as the PMIP-T concept.

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1. Introduction

Genetically modified (GM) plants, developed for the production of food and/or feed, have been on the international market since the mid-1990s. In general a single transgene conferring agricultural advantage, such as insect resistance, herbicide tolerance or growth in otherwise troublesome conditions, has been added to the plant (Dunwell, 2005; Morin, 2008). Hybrids between two such GM plants, harbouring two of more transgenes, have also reached the market (Dunwell, 2005; Umekawa et al., 2006). In recent years also nutritionally improved GM plants with changes of benefit to the consumer have been commercially explored, such as oil-rich plants with healthier fatty acid composition and maize with a changed amino acid balance (Birch et al., 2008; Newell-McGloughlin, 2008; Truksa et al., 2006).

Since the early 1990s, and especially over the last decade, another type of genetically modified plants, engineered to produce non-food proteins or metabolites for industrial or pharmaceutical use, has found substantial commercial attraction. In many cases common food- or feed plant species such as potato, carrot, soy bean, rice, tomato and papaya provide production platforms for such products. Major incentives behind this development include well established cultivation technologies in agriculture and, for several among them, extensive knowledge on protein expression mechanisms and methodologies for plant transformation (Sparrow et al., 2005; Twyman et al., 2005). Within the scientific and regulatory literature this phenomenon is referred to as “biopharming” or “molecular pharming” and the plants are commonly designated “pharma plants” or “non-food/non-feed GM plants” (NFGM). They are typically modified to express anyone among a broad range of proteins, such as immunoglobulins, cytokines, enzymes and hormones or viral capsid proteins for the production of vaccines (Agarwal et al., 2008; Bouche et al., 2003; Hernandez et al., 2007; Jiang et al., 2007; Lou et al., 2007; Moravec et al., 2007; Richter et al., 2000; Sardana et al., 2007; Walmsley et al., 2003). Major incentives for an increased commercial interest in plant biopharming lie in its scalability and dramatically lower production costs for the raw material, relative to fermentation-type manufacturing procedures (Twyman et al., 2005). Furthermore, the plant cell physiology enables post-translational processing of complex mammalian proteins resembling that of human cells, which is essential to the authenticity of transgenically expressed human biosimilars (McCormick et al., 2008; Sparrow et al., 2007). Upon ingestion, certain NFGM plants, or components derived thereof, may exercise negligible or modest adverse effect on consumers or livestock animals, whereas other varieties may be clearly toxic or otherwise highly inappropriate in food or feed (Kirk et al., 2005; Sparrow et al., 2007; Twyman et al., 2005). Because of perceived difficulties to accomplish complete separation from food or feed chains at all stages of growth, harvest and transportation the development of such plants raises concern for inadvertent admixture of conventional foods and feeds with NFGM plant material or its processed products. In addition, there is a risk of vertical gene flow to domesticated or wild relatives. These potential hazards translate to an increased need for risk management of NFGM plants and products obtained thereof.

In this paper, we present a brief review on various regulatory challenges in relation to risk management of conventional GM and NFGM plants, with a broad view to human and animal health and the environment. Moreover, we propose and outline a novel detection, quantification and identification framework, specifically designed for NFGM plants. The aim is to substantially facilitate future monitoring and traceability of NFGM plants and products, i.e. to enable more straightforward, faster and cost-efficient monitoring relative to that reliant on standard GM event screening approaches.

2. Genetically modified plants in a regulatory context

Global guidelines for risk analysis and risk assessment of GMOs have been developed by the Codex Alimentarius Commission (CAC) in several documents. One of those, The Principles Document, advocates that a new GM food should be assessed for its safety by comparing it with a food with an established history of safe consumption, in order to identify potential hazards requiring further considerations. This view is typically referred to as the Concept of Substantial Equivalence. The aforementioned document also stresses that risk managers should take into account uncertainties identified in the risk assessment and implement appropriate measures to manage them (Codex, 2003). The CAC guidelines are internationally endorsed, although the GMO authorisation procedure differs across national jurisdictions, where such are in place.

Within the European Union (EU) at present seven Directives or Regulations govern the use of GMO in areas pertaining to food and feed (Table 1). The three central legal frameworks directly related to food and feed are Council Directive 2001/18/EC on the deliberate release into the environment of genetically modified organisms, Regulation (EC) No. 1829/2003 on genetically modified food and feed, and Regulation (EC) No. 1830/2003 pertaining to the traceability of genetically modified organisms and the traceability of food and feed products, produced from genetically modified organisms (EC, 2001, 2003a,b). The first two of these legal instruments stipulate a pre-market assessment of GMO and aims at securing market release only of those GMOs that are safe for humans, animals and the environment. A regulatory body, designated the Community Reference Laboratory for Genetically Modified Food and Feed (CRL-GMFF) and being part of the European Institute for Consumer Health and Protection within the European Commission’s Joint Research Centre (JRC), was established under Regulation (EC) No. 1829/2003. Its main commitment includes scientific assessment and validation of detection methods for GM food and feed, which is conducted in collaboration with a network of European national control laboratories, assembled in the European Network of GMO Laboratories (ENGL). Prior to authorisation of any GMO in the EU, the applicant must present methods for detection, sampling and identification of the particular GMO event as well as samples of the food/feed and associated control samples.
For a review on GMO legal frameworks within the EU and elsewhere see (Paolelli et al., 2008).

Recently, the GMO panel of the European Food Safety Authority (EFSA) GMO-Panel published a Guidance Opinion for risk assessment of genetically modified plants used for non-food or non-feed purposes (EFSA, 2009). This document suggests that principles and practices for the risk assessment of NFGM plants should be analogous to those of ordinary GM food and feed plants, and that the degree of its unsuitability in food or feed shall dictate the degree of agricultural containment and, where appropriate, other risk-reduction measures. Three risk levels are suggested – one assigned as low/negligible and being devoid of requirement for confinement and two higher levels, each tied to a proportionate containment practice. Moreover, a two-step risk assessment for NFGM plants is suggested; one based on exposure assessment excluding confinement measures and one (whenever applicable) which incorporates segregation method(s) proposed by the applicant. It is further assumed that the cultivation of non-food/non-feed GM plants within the European Union – except for few cases – will go beyond contained use of GOMOs. Thus, Directive 2001/18/EC, i.e. the deliberate release into the environment, will be a major regulatory framework.

3. Monitoring GM food/feed traits

GM plants may be developed to express either a single new protein or several proteins. The latter GM plants, gradually becoming more common, may be produced through various procedures, involving simultaneous or sequential introduction of several identical or dissimilar transgenes into the host genome, or by conventionally crossing GM plants, each parental strain carrying a distinct genetic modification. In the former case, the modification cassette typically – but not consistently – appears as a single DNA sequence insert, whereas sequential-type operations or genetic crossing involve transgene integration in separate loci, referred to as stacked events (Halpin, 2005; Holst-Jensen et al., 2006; Taverniers et al., 2008). In food samples, there are currently no technical means to clearly distinguish a mixture of different transgenic varieties from a single stacked event (Holst-Jensen et al., 2006).

Although several experimental PCR formats have been described to identify and quantify GM events in food or feed (Akiyama et al., 2007; Wu et al., 2007; Yang et al., 2005, 2006, 2007; Zimmermann et al., 2000), quantitative real-time PCR (Q-PCR) has emerged as the prevailing method due to its specificity, wide dynamic range, speed, user-friendliness and its power to accurately quantify small traces of GM derivatives in processed material. A variety of technical platforms and Q-PCR variants, featuring a basically similar outline, are available (VanGuilder et al., 2008). They operate by continuously monitoring gradually increasing amplicon numbers, as enabled by recording fluorescence from intercalating dyes or fluorescently labelled reporter probes (BuhGasparic et al., 2008; Salvi et al., 2008). Assessment of GM sample quantity is usually made by a relative quantification in which the ratio between the transgene and an endogenous control gene is determined (Elenis et al., 2008). The endogenous control is preferably derived from a carefully selected endogenous and taxon-specific DNA sequence from the original plant, which is amplified either separately or jointly with the reporter motif.

A steadily growing number of licensed GM plant events have, however, created a need to develop methods for simultaneous analysis of many GM events. A series of such formats of variable complexity with good overall performance have been reported (Marmiroli et al., 2008; Michelini et al., 2008). Amplicons derived from multiplex PCR reactions are regularly detected and (in some cases) quantified subsequent to hybridisation to low-density oligonucleotide microarrays (Germini et al., 2004; Onishi et al., 2005; Rudi et al., 2003; Schmidt et al., 2008; Xu et al., 2007; Zhou et al., 2008). This analytical setting also furnishes for the concomitant probating of targets across the entire range of specificity classes (Leimannis et al., 2006; Tengs et al., 2007). It must be kept in mind, though, that albeit enabling higher throughput than singleplex settings, the multiplex concept cannot tolerate very large numbers of simultaneous amplifications in each individual test tube. Moreover, the joint multiplex/microarray format entails significant analytical expenses, since one chip per examined sample is needed. It is also highly inflexible, and an expanding number of licensed GM plant events would need constantly updated and revalidated arrays. Lastly, there are still remaining sensitivity and robustness challenges to confront (Marmiroli et al., 2008). The so-called padlock probes have been shown to enable an outstanding degree of multiplexing, thereby disclosing great potential for the simultaneous interrogation of thousands of genetic positions, such as single nucleotide polymorphisms (Hardenbol et al., 2003; Nilsson et al., 1994). To our knowledge this format has, however, not yet been applied to GMO detection.

Needless to say, it is advantageous to devise the simplest possible laboratory format to avoid potential analytical problems and to keep the expenditures low. For NFGM plants, of which some may impose significant potential health risks to consumers and livestock animals, there is currently a timely opportunity to introduce a much simplified monitoring system, relative to that currently applied to food/feed GM plants. In this paper we propose mandatory tagging of NFGM plants by means of a short DNA segment, holding both generic and specific motifs. To substantiate the concept’s usefulness we also delineate an assay based on a single tube Q-PCR reaction for detection and quantification of large numbers of NFGM plants, followed by a short-range DNA sequencing step for event specific identification. An outline of a suggested general design of such a surveillance framework is presented below, following the GM/NFGM review and discussion sections.

4. Plant molecular farming

4.1. Overall prospects for biomolecular production in plants

The production of peptides and proteins for technical or pharmaceutical purposes by expression in either bacteria or eukaryotic cells is well established for research and commercial production,
which can be optimised to give raw material of good quality. Nonetheless, GM plants also enable high-quality raw material with an even higher yield potential as well as modest manufacturing expenses, relative to expression in the aforementioned hosts (Giritich et al., 2006; Lienard et al., 2007; Ma et al., 2005b; Woodard et al., 2003). In the literature, these compounds are typically referred to as either industrial and pharmaceutical products (IPPs), recombinant plant-derived pharmaceuticals (PDPs), plant-made pharmaceuticals (PMPs) or, more specifically, plant-made vaccines (PMVs) (Kirk et al., 2005; Ma et al., 2005a,b; Mascia and Flavell, 2004). Actually, most products of heterologous expression in plants, either in pipeline for being marketed or already licensed, are pharmaceuticals (Lienard et al., 2007; Spok et al., 2008). Since the discussion in this paper encompasses a broad utilisation range of heterologously expressed plant substances, we have coined a new acronym – PMIP – derived from Plant-Made Industrial or Pharmaceutical products, a designation used henceforth.

The establishment of stable plant transformants can be accomplished by transgene incorporation in either the nuclear or the chloroplast genome. The former approach allows for post-translational protein modifications that are largely equivalent to those of mammalian cells (Faye et al., 2005). There are, however, subtle differences between the composition of mammalian-type asparagine-linked complex-type N-glycans and the corresponding carbohydrate attachments to proteins in plants. Research is, though, ongoing to equip plant cells with human-equivalent carbohydrate modification machinery, in part through heterologous expression of mammalian glycosyltransferases (Faye et al., 2005; Lienard et al., 2007). Integration of transgenes into the chloroplast genome enables very efficient expression of the transgene-encoded protein (Daniell et al., 2002; Staub et al., 2000). However, proteins expressed in this way, contrary to those expressed in the nucleus, are devoid of mammalian-type post-translational modification, which limits the range of proteins of commercial interest that can be produced (Daniell et al., 2005a). Recombinant proteins can also be produced using transient expression from plant viral vectors. The first generation essentially encompasses functional viruses that express the coding sequence of the protein of interest. In a recently developed second generation of plant viral vectors only the viral elements required for efficient expression of the transgene are maintained. These vectors clearly limit the risk of lateral or systemic dispersal, but require infection of Agrobacterium for delivery of transgenes into the cell (Gleba et al., 2007).

Not only are plants highly effective biological factories for the manufacture of proteins, but food plants also have all parts of the agricultural process, including sowing, irrigation, harvesting, chopping and downstream processing of the raw material developed to considerable sophistication. As a consequence, there are relatively low expenditures for fabrication of the raw material and very good scalability. Moreover, no known human pathogen is inherent to plants (Twyman et al., 2005). These circumstances comprise an overall high potential to fulfill increasing demands on the production of therapeutic antibodies, vaccines and other bio-molecules, which cannot readily be met through current fermentation methodology (Daniell, 2006; Lienard et al., 2007; Ma et al., 2005b). For example, vaccines against hepatitis B virus and rabies virus, currently produced in yeast cells, are manufactured in insufficient supply for the needs in many developing countries (Ko and Koprowski, 2005). The overall yield improvement, as accomplished by expression of various recombinant immunoglobulins and other heterologous proteins in plants, may not only supply an adequate quantity but also allow new applications, such as topical (mucosal) antibody application, e.g. to counter dental caries progression, or to prevent venereal pathogen transmission (Key et al., 2008). Due to these potentials to conquer new markets, NFGM plant agriculture is likely to expand considerably within a reasonably near future, both regarding diversity of plant species used as production platform and acres allocated to their cultivation.

Cultivated plants availed as expression systems for recombinant proteins encompass mainly seed and vegetable food plants such as maize, rice, soybean and barley as well as potato, tomato and carrot. They also encompass leaf plants, such as lettuce, and, particularly, the non-food plant tobacco (N. tabacum). Seed-producing plants have, however, several specific advantages, which mainly lie in these plant’s already established systems for efficient protein production and deposit. The seeds are indeed ideal for storage of newly expressed proteins, since this environment, due to natural requirements, is highly protective against protein degradation (Stoger et al., 2005; Twyman et al., 2003). Transgene-encoded proteins are thus readily amenable to enrichment and further purification by means of standard methodology, e.g. extraction and chromatography (Kusnadi et al., 1997; Menkhaus et al., 2004).

4.2. The current pre-market and market situation

The last several years have witnessed a staggering increase in the number of plant-made biologicals, including a broad range of human biosimilar compounds. Many among those are ultimately aimed at pharmaceutical use, being either cytokines/growth factors (e.g. interferons and various interleukins, somatomotropin, erythropoietin) or enzymes (e.g. acetylcholinesterase, transglutaminase). Notably, vaccines (e.g. choleliverinin B subunit, Norwalk virus capsid, G protein of rabies virus) have emerged as a relatively large PMIP assembly (Daniell, 2006; Lienard et al., 2007; Ma et al., 2005b; Spok et al., 2008; Twyman et al., 2005). An additional and very large group embraces a wide collection of immunoglobulins, notably monoclonal, mouse/human chimeric or other antibody varieties (Lienard et al., 2007; Twyman et al., 2005). Already as of 2006 a considerable number of PMIPs, including oral vaccine against Hepatitis B, human intrinsic factor, gastric lipase, human lactoferrin and human lysozyme, had entered clinical trial phases and are thus approaching commercialisation (Spok, 2007). In 2007, a plant-derived veterinary vaccine, manufactured by Dow AgroSciences, was granted market licence, thereby being the first regulatory approved PMIP of truly pharmaceutical character. Later in the same year, Cuba’s Biotechnology and Genetic Engineering Center received regulatory approval to market a PMIP-derived human antibody against Hepatitis B virus (Sparrow et al., 2007). Indeed, molecular farming has been suggested as likely to become the next major commercial venture in biotechnology and, already in 2004, an EU-funded academic consortium – Pharma-Planta – was established to address pertinent issues, including those of biosafety (Sparrow et al., 2007). Other sorts of notifications/applications pertain to specifically coloured carnations and a starch-modified potato called AMFLORA, the latter variety being in the EU approval system since spring 1996, yet without any final decision.

5. Containment measures for NFGM plants

Using food and feed plants as a platform for production of technical, diagnostic or pharmaceutical products involves an apparent risk of adventitious adulteration of food/feed commodities or the environment with PMIP-producing plants or their traits. There are many potential routes to admixture. For example, neither regular grain transport vehicles, nor grain milling facilities, used in food production are typically designed for complete clean-out. The use of the same equipment, therefore, constitutes a hazard. Thus, each step of PMIP production, including on and off site transportation, grain-handling, drying, storage and downstream
processing operations of plant material must be strictly planned and carefully conducted to arrive at a reasonably safety level of protection against contamination of commodity food plants (Mascia and Flavell, 2004; Sparrow and Twyman, 2009; Twyman et al., 2005). Hence, prescription of NFGM management strategies, including those pertaining to containment, needs guidance by scientifically justified criteria and judgement on a case-by-case basis. Segregation measures roughly fall into three main categories: physical isolation, natural confinement and genetically engineered containment.

5.1. Physical isolation

The most direct method to reduce accidental pollen flow and seed dispersal is physical segregation of the NFGM plants from the environment. Examples of such contained production facilities include greenhouses, growth cabinets and underground chambers (Dunwell, 2005).

5.2. Natural containment

For plants grown under conventional open-field agricultural conditions, geographical areas where neither domesticated varieties of the plant species nor their wild relatives grow can be used to minimize gene flow. Barrier plants planted at the field boarders may further reduce untoward cross pollination. Several PMIP-expressing commodities are based on plants currently not used as food and feed, for example tobacco (various Nicotiana species), thereby significantly reducing the risk of contaminating commodity crops (Daniell, 2006; Murphy, 2007; Sparrow et al., 2007). Cleistogamous plants, which accomplish self-pollination within a closed bud as platform for protein production, offer yet another inroad to protection against dispersal of PMIP-transgenes into the environment.

A general precautionary method to restrict seed dispersal is to harvest plants prior to flowering, which could be applicable to leafy crops such as alfalfa, spinach and tobacco. However, comparatively fast proteolytic degradation of proteins in leaf tissue subsequent to harvesting renders this approach less favourable, compared with techniques based on seed-directed expression (Streatfield, 2005). When plants are grown to maturity, adequate measures to eliminate volunteer plants the following season must be exercised. In general all strategies relying on natural containment are leaky, i.e. a risk of horizontal gene flow remains (Dunwell, 2005).

5.3. Genetically engineered containment strategies

Molecular engineering approaches to control gene flow from transgenic crop plants have hitherto mainly been focused on maternal inheritance, male sterility and seed sterility. Several other containment strategies, such as fruit or pollen specific excision of transgenes and transgenic mitigation, have also been proposed to control gene flow (for review see Daniell (2002), Dunwell (2005), Murphy (2007)).

Maternal inheritance has been obtained by introgression of transgenes into the genome of chloroplasts, which are almost exclusively maternally transferred. The risk of transfer and establishment of transgenes from chloroplast DNA to other plants via pollen is thereby much lower than for those located in the nuclear genome (Daniell, 2006). Formation of hybrids by fertilization with pollen from wild plant species is, however, not prevented using this strategy. Male sterility, as a result of disrupted pollen development, can be achieved in several ways. One approach involves transfer of genes controlled by tissue- or cell-specific promoter elements and coding for proteins such as site-specific nuclease or ribonucleases (Khan, 2005; Kurek et al., 2002; Mariani et al., 1991). Another example of genetically engineered male sterility, implemented in several different species, is a pollen-destructive system that acts by means of the noxious ribonuclease (barnase) (Baroux et al., 2001). When combined with a function-restoring mechanism, provided by the barstar gene, plant fertility can be restored (Kobayashi et al., 2006). Similarly, seed sterility has been obtained through the introgression of genes resulting in conditional lethality using the Genetic Use Restriction Technology (GURT) (Visser et al., 2001). An interesting development of the molecular mechanism underpinning GURT has found application in the Gendeletor technology, in which transgenes can be excised from the host genome in a pollen and seed specific manner by means of site specific recombination, rendering offspring of the NFGM plants void of any transgene (Luo et al., 2007).

Yet another genetic containment strategy, aimed at preventing introgression of transgenes into wild populations, is designated transgene mitigation. Briefly, the technique relies on a tight linkage of the desired gene to another element, the latter conferring a selective disadvantage to the plant in the wild but not during agriculture (Al-Ahmad et al., 2005). However, this methodology is not strictly designed to prevent escape of transgenes, but rather renders its survival in the environment with wild plant populations less favourable.

5.4. Overall efficacy and consensus practices

Molecular containment technologies continue to develop gradually to higher levels of sophistication and efficiency. No single strategy has yet, however, proved broadly applicable across all crop species (Daniell, 2002). Presumably, a scientifically justified combination of confinement/mitigation methods, applied to each NFGM plant, may provide the best possible direction to attempt compensating for deficiencies of any one alone. Nonetheless, the current situation is characterised by considerable technological heterogeneity and a limited number of acknowledged genetic containment methodologies, all with comparable and high level of protection against NFGM gene flow, is currently relatively remote (Murphy, 2007). Reassuringly, issues on PMIP pertaining to food/ feed safety and environmental integrity are relatively intensely discussed in the literature and are also a clearly developing regulatory area (Kirk et al., 2005; Peterson and Arntzen, 2004; Sparrow and Twyman, 2009; Sparrow et al., 2007; Streatfield, 2005; Twyman et al., 2005). Scientific and regulatory consensus of such preventive measures is, however, not likely to appear anytime near. Already in 2005, Dunwell and Ford concluded that the available methods cannot guarantee full protection against transgene escape, inferring from this that continuous monitoring is needed (Dunwell, 2005). We believe this stance on NFGM plants still holds full relevance.

6. Biopharming with food/feed plants: a foolproof practice?

Due to the specific quality of most NFGM plants, the seed material will be handled and transported separated from other seed materials, and the cultivation, although essentially comparable to that of food and feed plants, will be on contract and presumably entailed to significantly enhanced surveillance. As the heterologously expressed molecular constituents of NFGM plants ultimately will become refined industrial reagents or pharmaceuticals, downstream handling of the plant materials are expected to differ from that of food/feed. Nonetheless, the phenotypic similarity of such plants to those of food and feed, whenever the latter group of species serves as hosts for biomolecular production, poses an obvious challenge to the trust of consumers, retailers and
farmers of food-producing animals. Moreover, a risk of non-authorised channelling of residual biomass (left-over after biomolecule extraction) into the food/feed chain might arise from expenditures connected with its disposal. Another issue pertains to risk for occupational toxicity. In case of the tobacco plants, well known to produce the toxin nicotine, field workers often develop “green tobacco sickness”, especially if harvesting wet, uncured leaves. Exposure to the toxin occurs mainly via the dermal route from fluid on leaves, but may also stem from aerosol inhalation (Andersson et al., 2003). These routes of exposure, conferring toxic insult, are conceivable also for PMIP-producing plants. Albeit important this topic is not further elaborated on in this paper.

A relaxed attitude to the risks associated with biopharming is occasionally seen in the literature, and it is typically linked to one or more of the following two views: (i) heterologous PMIP expression by NFGM plants most often involves proteins that upon accidental ingestion are readily degraded to innocuous peptides or amino acids; (ii) there are already agricultural and technological practices in place, i.e. physical, natural and sophisticated genetic containment measures as well as adequate distribution management practices to prevent accidental admixture of NFGM plants with those destined to food or feed (Goldstein and Thomas, 2004; Ma et al., 2005b). It may thus be argued that accidental exposure to such NFGM plants, or processed products thereof, is of modest concern because of protein decomposition in the gastrointestinal tract. This attitude may be justified in several cases, but certainly falls short of supporting a norm. Actually, gastric pH and proteolytic activity in the gastrointestinal tract depend on several factors such as age, diet and medication. There is furthermore a strong inducement within the medicinal sector to exploit established as well as novel avenues to oral medication of peptide and protein pharmaceuticals. Actually, immunization through oral administration of PMIPs, subsequent to refinement into defined and dose-adjusted pharmaceutical preparations, holds potential as a key future use for PMIPs (Daniell et al., 2005b; Ma et al., 2005b). Considerable pre-clinical progress in the area has been made lately. For example, mice fed plants expressing a cholera toxin B/green fluorescent protein fusion complex (CTB-GFP) were shown to carry this intact complex in liver and spleen (Limaye et al., 2006). Moreover, oral administration of a multi-component vaccine was demonstrated to confer protection to mice against enteric disease (Yu and Langridge, 2001). These observations indicate that protein integrity can be reasonably well protected against acid- or protease-mediated degradation. Actually, induction of tolerance to either viral or bacterial agents, or otherwise unintentional immune responses, due to adventitious adulteration of the food/feed chains have been underlined as especially problematic potential NFGM-associated scenarios (Kirk et al., 2005; Streetfield, 2005). Thus, there are special potential risks associated with certain applications of biopharming, which all need dedicated risk assessment and risk management practices.

How likely is accidental contamination of the food and feed chains with components derived from NFGM plants? Several episodes of accidental admixture of conventional food plants with non-approved GM food plants have indeed been reported. An especially notorious example of poor segregation management occurred back in 2000, involving a transgenic variety of maize designated CBH 351, also known as StarLink™ maize (Bernstein et al., 2003; Raybourne et al., 2003). This GM maize event expresses a mutant Cry9C insecticidal protein (originally from a Bacillus thuringiensis gene), and was approved by the FDA in the USA for feed purpose only, because of concern for potential allergenicity of the insecticidal protein. However, widespread admixture of maize grain used as food with this transgenic event was identified in the USA, as well as in exported maize and maize-products. Whilst subsequent studies failed to support initial fears of ampler allergenicity, the StarLink™ contamination scandal nevertheless sent shockwaves through food regulating communities and scared consumers (Bratspies, 2004; Teshima et al., 2002). Another example of accidental exposure to a non-approved GM product was reported in 2006, and was related to the herbicide-resistant Liberty Link 601 (LL RICE 601) transgene rice variety grown in field trials from 1998 to 2001, originally by Aventis Crop Science but subsequently taken over by Bayer. In 2006 LL RICE 601 rice was detected in conventional rice at Riceland mill in Arkansas, which assists all major rice-growing states in the USA. This incident, involving a GMO with an official risk assessment, ultimately resulted in transient import bans of US rice to Japan and the EU (Hishaw, 2007). Moreover, an insect-resistant Chinese rice variety, commonly referred to as Bt 63 and modified to express a Cry1Ac gene (originally derived from the soil bacterium B. thuringiensis) appeared in food on the EU market on several occasions in 2006 and 2007, without being authorised as food in the EU (Huggett, 2008). A fourth international controversy, referred to as the Prodigene incidents, arose in 2002 when volunteer NFGM maize plants expressing a veterinary vaccine appeared in conventionally cultured soybean, the field used in the previous season to cultivate the NFGM maize (Fox, 2003). In one case close to 18 million litres of harvested soybean had to be impounded, due to contamination. All of the above-mentioned episodes of adventitious mixing of conventional foods with non-approved GM food or NFGM plants illustrate inadequate risk management and have resulted in several filed lawsuits against the responsible parties (Fox, 2003; Hishaw, 2007). Although it is unlikely that human or animal safety was drastically challenged by the incidents described above, the Prodigene episodes created considerable concern regarding the safety of biopharming PMIP-producing plants. These and other management failures of GM or NFGM plants not mentioned here have triggered an intensified debate on the need for stricter regulatory oversight to ensure integrity of the food/feed chain (Fox, 2003; Sparrow et al., 2007).

It is our contention that extensive usage of major commodity food plants as platforms for production of raw materials for the biotechnology and pharmaceutical industrial bodies is very likely to ultimately result in episodes of accidental contamination of foods or feeds. The likelihood for adulteration would increase in proportion to the number of acres allocated to NFGM growth. The admixture might occur at several stages; during cultivation, seed production, harvest, transportation and subsequent distribution and processing (Sanvido et al., 2007). Additionally, risk for food and feed chain adulteration by plant residues must be seriously considered. Furthermore, plant remnants in field might give rise to considerable environmental challenges.

7. PMIP vigilance: choosing a methodological strategy

7.1. A need for expedient and reliable surveillance of NFGM plants and PMIP

We feel that a clear demarcation line lies between plants cultivated with the intention to produce food and/or feed (non-GM or GM), and those destined for the production of raw material with an industrial, diagnostic or pharmaceutical purpose. Apart from prescribed containment practices, we find it prudent to specifically emphasize this distinction in a regulatory surveillance context. Hitherto, considerable efforts have been devoted to containment-oriented risk management practices for PMIP-producing plants, aiming at the prevention of food/feed admixture with these products. Much less energy, however, has been allocated to methods aiming at detecting such adulteration. Nonetheless, a need for reliable systems to probe the efficiency of various containment practices can clearly be identified.
7.2. Protein versus nucleic acid–based NFGM detection

A dominant seed colour marker for PMIP plants has been suggested in the literature (Dunwell, 2005; Sparrow and Twyman, 2009; Streafield, 2005). Already in 1999 green fluorescent protein was proposed as a visual marker of transgenic plants, and purple maize was subsequently demonstrated as an example (Commandeur et al., 2003; Harper et al., 1999). Actually, a detailed description of a system alongside the latter direction, based on macroscopic detection of red fluorescent protein, was recently reported (Rademacher et al., 2009). As suggested by the authors, the presence of a tightly linked transgene may confer visual identification of PMIP features with high sensitivity, using low technology equipment (EFSA, 2006; Rademacher et al., 2009). Indeed, this setting has some rather appealing features, in particular prompt detection by means of unpretentious analytical instrumentation, subsequent to none or very simple sample preparation. Nonetheless, a visual marker approach is inherently reliant on the expression of proteins, which are prone to undergo denaturation upon heating or otherwise harsh treatment. Thus, contrary to nucleic acid–based methodology, it may not be suitable to control processed food or feed. Further, our strictly DNA-founded PMIP-T concept (see below) incorporates both generic and specific information, the latter being outside the scope of visual identification. Lastly, the Q-PCR methodology, being a core part of detection/identification through PMIP-T, enables an analytical sensitivity which the fluorescent protein assay format cannot match. Seemingly, however, the visual detection methodology – as also stressed by the authors – holds considerable potential for practical usage when applied to field work to facilitate GM/NFGM plant breeding programmes (Rademacher et al., 2009).

7.3. Enhanced NFGM monitoring

The methods applied, in a regulatory context, to monitoring presence of GMO or processed products thereof in food and feed are almost consistently reliant on nucleic acid (DNA) detection, by means of the Q-PCR principle (Marmiroli et al., 2008). Tests for presence of GM material can aim at different levels of specificity. DNA sequences encompassing parts of the heterologous gene and translocating into host genome are typically used to ultimately define a detected GMO event (Holst-Jensen et al., 2003). As briefly outlined above, microarray-based multiplex analytical settings have also found application within this area, to simultaneously analyse several transformation events (Peano et al., 2005; Hamels et al., 2007). Each of these format types has its own merits and restrictions. We feel, though, that a straightforward and cost-efficient screening setting is particularly needed for NFGM plants because of the special risks attached to them. Such methodology can be built on already established technology for GM-screening and identification, but also incorporating additional parts, which jointly would both simplify and enhance NFGM plant surveillance.

8. Proposal for improved risk management of NFGM crops through targeted PMIP vigilance

In order to enable detection of NFGM plant material, with high sensitivity, in both non-processed and processed food and feed, and to comply with already established food and feed control practices applied to screening for presence of GM material, we suggest a vigilance system for PMIP-expressing NFGM plants be based on nucleic acid detection. As new GM plants, including those of the NFGM type, are continuously approved by the Authorities responsible for food control, there is a need to screen an increasing number of different GMOs. Moreover, the future will conceivably reveal an escalating molecular heterogeneity of transgene constructs, translating to an intricate analytical situation. On the assumption that the current GMO-analytical course is maintained, this development will impose vastly escalating costs and increased risk for experimental errors (Cankar et al., 2005, 2008).

In this paper we therefore propose a screening-based surveillance strategy based on a generic principle, rather than on the established practice for event-specific DNA sequence targeting, but still holding potential to carry specific information in a very compact appearance. Thus, the concept envisages a sustainable future vigilance practice for all NFGM plants below a rather strict safety threshold to be licensed for growth and/or marketing. To our perception the suggested tagging framework would principally apply to almost all current and future NFGM plants. This new inroad to regulatory oversight is, however, not aimed at the replacement of current EU requirements for GMO traceability and the entailed screening exercises. Adoption of the PMIP-T strategy would, though, require extension of pertinent parts of the current legal framework.

8.1. General outline

In essence, we believe a mandatory implementation of a molecular NFGM plant specific DNA identification tag, in this paper designated PMIP-Tag (abbreviated PMIP-T and outlined below), would enable an efficient vigilance-based risk management practice for this type of GM plants, without imposing any significantly added expenses on commercial plant breeders. This suggestion solely stems from an incentive to improve consumer and livestock safety by promoting regulatory consistency in conjunction with analytically high quality surveillance. The presence of a PMIP-T fragment would allow very simplistic and relatively inexpensive identification and quantification of NFGM plants or any food/feed products derived thereof. If properly designed, a PMIP-T would enable a single-tube/single-reaction procedure for detection of any sort of NFGM plant, using only a single and universally applicable pair of PCR primers (Fig. 1). This set up can easily be translated to concurrent identification and quantification by Q-PCR, regardless of NFGM event features in other respects. Actually, a duplex Q-PCR reaction, encompassing generic PMIP-T PCR primers and those of an endogenous reference gene, allows for single tube-based quantification. Furthermore, if traces of any NFGM plant are found, a single sequencing reaction encompassing about 10–20 nucleotides, would provide all necessary identification details on its origin and character.

8.2. Detection of NFGM by means of PMIP-T

The implication of this proposal is that (almost) any NFGM plant modified to produce PMIP is obliged to carry a PMIP-T DNA fragment as a specific and unique identifier. The PMIP-T genetic identifier should preferentially be part of the NFGM transgene construction, or otherwise occur in a transgene-proximal location, to minimize the risk for segregation from the gene of commercial interest. Moreover, it is essential to keep the identifier relatively short, tentatively about 100 nucleotides, to maintain maximal preservation of the fragment during food/feed processing, to support PCR amplification efficiency and reduce the time needed to accomplish a reaction and to minimize any molecular design complexities imposed on commercial plant breeding parties. Notably, the PMIP-T fragment is intended to appear as a completely silent piece of DNA, which will neither itself code for any RNA/mRNA, nor act as an initiation, enhancing or silencing motif for transcription of closely located DNA sequences. Thus, the overall PMIP-T layout should be unique enough to fulfill the necessary requirements for accurate NFGM detection, quantification and identification, but essentially devoid of biologic activity.
The PMIP-T fragment consists of two major sections (Fig. 1), each corresponding to a distinctive analytical procedure designated 1st and 2nd scheme, respectively (Fig. 2). Firstly, the border regions of the PMIP-T fragment are generic for all NFGM plants and are designed to match a single pair of universal PCR primers. These segments must be designed with utmost care, aiming at keeping the risk of non-specific amplification at a minimal, preferably non-existent level. Exhaustive bioinformatic screening of public sequence databases should assure low risk for false priming and potential spurious screening results. A generic Q-PCR probe target sequence, complementary to the sequence of a fluorescent reporter molecule such as a Taqman or molecular beacon probe, is also a key part to incorporate into PMIP-T. Using these PCR primers and a reporter probe in a Q-PCR analysis would thus identify whether the tested food/feed is a NFGM crop or contain residues thereof, and – in the event of positive test readout – the extent of adulteration (1st scheme). This experimental outline is altogether analogous to that of the established practice for GM food/feed screening, except for the essential difference of being reliant on standardized universal PCR primers. Secondly, the core PMIP-T part is unique to each individual NFGM event and easily decoded by e.g. short-range DNA sequencing on the PCR product produced in the initial Q-PCR reaction (2nd scheme). Again, a generic reagent – appearing as a single standard (universal) sequencing primer – is proposed in order to simplify the analytical procedure.

We suggest the 2nd scheme analysis be founded on a short nucleotide sequence tag, based on the quaternary numeral system, which is well adapted to representation of the genetic code by DNA. A code format based on this system, carried by a DNA tag encompassing just eight nucleotides, would principally enable specific identification of each among about 65,000 varieties. Within the EU, an accordingly coded sequence tag may easily be designed to conformity with the legally required assignment format for GMOs (EC, 2004). Detailed knowledge of any NFGM would thus be made promptly available by means of two currently very established and accessible molecular biology technology platforms: PCR and short-range DNA sequencing. A key premise of the latter part, however, is that a mandatory filing of each such code in a repository, preferably under the auspices of an appropriate Authority. In the EU the most rational place to hold and curate such information is The European Commission’s Joint Research Centre (JRC). The aforementioned body already holds the required molecular biology documentation for each GMO being approved for marketing within the Community as well as a publicly accessible GMO Methods Database.

Assuming a positive 1st scheme experimental readout, an Authority may already at this stage initiate early risk management actions, such as food/feed batch tracking. Sample(s) found to harbour NFGM crop residues would then undergo characterisation, according to the ensuing scheme, ultimately revealing their identification. The PMIP-T layout permits NFGM plant decoding by virtue of short-range DNA sequence determination, quickly achievable by automated instrumentation such as the Pyrosequencing system, which can provide a sequence of 20–40 nucleotides within less

![Diagram](image_url)
than an hour (Alderborn et al., 2000; Ronaghi et al., 1998; Ronaghi et al., 2007). Principally, the identification code can also be deciphered by means of hybridisation to a molecular barcode microarray (Pierce et al., 2006). Hence, a reasonably well equipped laboratory would thus rather promptly provide the ensuing 2nd scheme assessments, in response to 1st scheme readouts, thereby eliminating unnecessary delay in further risk management exercises. Results from both of these analytical formats are essential to guide well-tuned further measures, since NFGM plants may express any one among a broad range of PMIPs, some of which posing modest challenge to consumer and animal health. Future regulatory work might arrive at a single limit or even multiple threshold levels for tolerance of such adventitious contamination.

Naturally, PMIP-T integrity in each accordingly labelled NFGM plant must be carefully preserved across generations. We believe that safeguarding DNA sequence conservation of this part would be feasible to incorporate in the respective manufacturer’s regular plant/seed quality assurance practices.

8.3. Dual-purpose GM plants

A composite food-feed/industrial avail of NFGM plants (referred to below as Composite Purpose GM plants, CPGM) is principally an exploitable commercial direction. Clearly, however, plant molecular farming can enable high yield of a primary bio-molecule but, conversely, it is difficult to reduce such compounds to insignificant levels in the residual biomass. Thus, a need to meet such requirements is likely to impose prohibitive expenses on production of substance(s) of industrial or pharmaceutical interest. Principally, a dual usage strategy may, though, possibly apply in the event edible oil can be extracted from seeds of such GM plants. This situation would, however, not disqualify the implementation of the PMIP-T concept, since such oils typically are devoid of detectable DNA and protein, a condition which must be rigorously met in those cases. Regarding NFGM plants, designed to produce bio-molecules for pharmaceutical refinement or for other industrial purposes, however, we believe such cases be exceptional at best. The risk of bioactive substance residues to appear in the plant left-over, thereby entering the food and feed chains, would require very extensive scientific documentation on the CPGM plant constituents in order to perform a complete risk assessment. It is even conceivable that risk managers would handle those plants as if they actually were traditional GM plants for the food and feed market. This management strategy would require a demonstration that residuals of the CPGM plants are essentially equivalent to an intact or regularly processed (non-modified) conventional counterpart.

9. Feasibility study in progress

The overall message in this paper is relatively straightforward, and the technical layout should rather easily be implemented in any molecularly qualified plant breeding laboratory. We have nonetheless embarked on an experimental course aiming at demonstrating a functional PMIP-T system, as appearing in a plant. The idea is simply to show the principal characteristics of such a generic system, designed to enable facile identification and quantification of NFGM plants and thereof derived material, which also allows for event-specific description within the same concept.

10. Conclusions

10.1. Perceptions of NFGM plants

A radical approach would be to either prohibit the use of food/feed plants for non-food/non-feed applications, or to handle NFGM food/feed plans equivalently to regularly cultivated GM food/feed plants and assess their safety on these premises. Such views may, however, be perceived as over-zealous, partly because there are many non-food/non-feed applications that neither pose significant risk to the consumer nor to animals fed the products, e.g. the AMFLORA potato with altered starch composition, grown in field trials in the EU (EFSA, 2006). Moreover, anyone of these stances would severely restrain production of comparatively inexpensive NFGM bio-molecules with obvious applications in various fields, including manufacture of biological pharmaceuticals. Another way forward would involve the definition of several risk-based boundaries, the principal one segregating NFGM crops, or thereof derived substances, that can be tolerated as contaminants in food/feed from those denied such permit. This direction may, admittedly, incur difficulties to define an appropriate demarcation line. In our opinion, the differentiation must be set at a strict level to provide protection not only against overt toxicity or immunological insult to humans and animals, but also against agricultural mismanagement of nutritionally inferior NFGM plants. Actually, potential harm to the environment should also be considered in this context. More subtle demarcation lines may apply within the NFGM category not endorsed as food/feed contaminant, which could help risk assessors to suggest appropriate agricultural containment schemes.

10.2. Consistency across risk assessment and risk management

Compulsory tagging of NFGM plants with a PMIP-T fragment would implement a demarcation between two principally different risk management practices. The one not relying on PMIP-T pertains to mandatory GMO monitoring and product labelling, as already defined in the EU legislation, to assure product identity, safety and regulatory compliance, thereby supporting the consumer’s possibility to make choices between various foods on the European market. The other practice would, as suggested in this paper, be based on PMIP-T tagging and strictly relate to consumer and livestock animal safety. Thus, the PMIP-T DNA concept can be considered as an instrument to check efficiency of the confinement measures adopted in the risk management decision process, in response to judgements based on risk assessment, and to allow risk managers to take prompt actions in cases of failing containment. Separation of NFGM plants from those aimed at food or feed also feeds into two principally dissimilar regulatory considerations: (i) judging whether a crop is safe enough to be licensed as a GM food or feed, and (ii) to evaluate potential hazards to consumers and the environment to support decisions on appropriate agricultural segregation.

10.3. Expedience through simplicity

A technically advantageous analytical outline for NFGM detection will become increasingly important when agriculture of such plants becomes abundant, a situation likely to appear in many areas worldwide within a relatively near future. Surveillance of NFGM plants or derived products thereof, based on the proposed PMIP-T, is inherently adapted to very low experimental complexity, by being reliant on regular and largely automated equipment and may thus be conducted without unnecessary expenditures. Thus, the generic PMIP-T fragment is designed to allow expeditious screening, including prompt identification and quantification of NFGM plants or parts thereof in the event they should accidentally appear in food or feed. Compared with either a Q-PCR involving an event-specific pair of primers in each reaction, or a multiplex-based amplification system combined with an oligonucleotide microarray, the PMIP-T concept compares favourably with respect to simplicity, robustness, speed and expenditure. A positive assay
signal already in a single PCR reaction would permit prompt initial risk management actions. Further measures may follow from complete NFGM plant identification. Thus, the principal incentive to the generic molecular tagging concept, as outlined in this paper, is to enable efficient, expedient and relatively inexpensive NFGM plant-monitoring, which can be incorporated within a standard control program.

Conflict of interest

The authors declare that there are no conflicts of interest.

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