



Assessment of GE food safety using ‘-omics’ techniques and long-term animal feeding studies

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Despite the fact that a thorough, lengthy and costly evaluation of genetically engineered (GE) crop plants (including compositional analysis and toxicological tests) is imposed before marketing some European citizens remain sceptical of the safety of GE food and feed. In this context, are additional tests necessary? If so, what can we learn from them? To address these questions, we examined data from 60 recent high-throughput ‘-omics’ comparisons between GE and non-GE crop lines and 17 recent long-term animal feeding studies (longer than the classical 90-day subchronic toxicological tests), as well as 16 multigenerational studies on animals. The ‘-omics’ comparisons revealed that the genetic modification has less impact on plant gene expression and composition than that of conventional plant breeding. Moreover, environmental factors (such as field location, sampling time, or agricultural practices) have a greater impact than transgenesis. None of these ‘-omics’ profiling studies has raised new safety concerns about GE varieties; neither did the long-term and multigenerational studies on animals. Therefore, there is no need to perform such long-term studies in a case-by-case approach, unless reasonable doubt still exists after conducting a 90-day feeding test. In addition, plant compositional analysis and ‘-omics’ profiling do not indicate that toxicological tests should be mandatory. We discuss what complementary fundamental studies should be performed and how to choose the most efficient experimental design to assess risks associated with new GE traits. The possible need to update the current regulatory framework is discussed.

Introduction

Safety assessment is structured, step-wise, and based on a comparative approach. The substantial equivalence concept according to the principles outlined in the Organization for Economic Cooperation and Development (OECD) consensus documents [1] encompasses a comparison of biochemical composition with a non-GE line considered to be safe. The GE line is compared to its near isogenic counterpart, according to specific determinants such as molecular characteristics, and agronomic and phenotypic traits [2]. Moreover, public consultation procedures have been established.

Despite the fact that an extensive and robust compositional assessment for evaluating the substantial equivalence of GE crop plants is currently imposed before market introduction (including current toxicological tests), some citizens remain sceptical of the safety of GE food and feed in the EU [3]. The first question is: may the improvement of a plant variety through transgenesis result in unintended effects which may be triggered by the insertion of a transgene? If so, could it impact on consumer and animal health? The second question regards the safety of animals: can long-term studies as well as multigenerational feeding studies detect potential unintended effects in animals? These questions have prompted new studies, carried out by public research laboratories using alternative evaluation techniques (i.e. not part of the regular evaluation process), namely high-throughput ‘-omics’ profiling of

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GE varieties and long-term animal feeding studies as well as multi-generational feeding studies.

The aim of the present study was to investigate the data of these articles in the context of detection of possible unintended effects in GE plants.

Materials and methods

Our database 'BergeRicrochGMlibrary' of studies using transgenic plants is built from selected references from journal sites (such as Ingenta, PubMed, WoK). Search terms include 'transgen*' OR 'transgenic' OR 'GM' OR 'GE' OR 'engineered' OR 'modified' OR 'genetic* engineer*' OR 'genetic* modifi*' OR overexpress* OR 'insect* resist*' OR 'herbicide tolerant' OR 'glyphosate tolerant' AND 'plant' OR 'species' OR 'organism' OR 'crop'. This database is maintained by the analyses of tables of contents of journals from collections such as BioOne, Elsevier, ScienceDirect, Springer, etc. (507 journals); by the monitoring of 352 journals not covered by these collections; and by the monitoring of 530 other journals [4].

To perform a systematic review using an evidence-based decision approach, we followed a decision tree (Flow of Included Studies). We selected relevant search terms to sort a set of publications from potentially relevant studies identified and screened for retrieval to studies included in a literature review. For this review, the references were sorted using search terms related to 'omics'; 'transcriptomics'; 'proteomics'; 'metabolomics'; 'long-term'; 'mutigenerational'; 'feeding studies', 'Food safety', and 'Feed safety', 'food and feed'. Then selected papers on feeding studies are sorted using EndNote6 with a search of 'feeding studies', 'feed', 'fed' terms in titles and in abstracts separately. Long-term studies, that is longer than 90 days, extending over most of the lifetime of the test species, are used to assess the potential chronic toxicity and/or carcinogenicity for single defined substances [2].

Results

High-throughput profiling studies

Recent developments in biotechnology include the emerging technologies of '-omics' – transcriptomics, proteomics and metabolomics. Transcriptomics measures the steady-state mRNA abundance from a given tissue source. Proteomics is a technology for both qualitative and quantitative analyses of proteins, and investigations into protein posttranslational modifications. Metabolomics refers to the complete set of metabolites synthesized through a series of multiple enzymatic steps from various biochemical pathways.

High-throughput '-omics' profiling techniques have been suggested as a nontargeted approach to detect unintended effects in GE plants. The application of proteomics in food science [5] can address the safety issue of food of various origins, including transgenic food, in parallel to the transcriptomic and metabolomic approaches. We examined recently published profiling studies concerning major crop plants [6]. Here we update the evaluation of GE crops, so in total 60 profiling studies were published. The over-arching objective of our investigation is to explore the possibility, or not, of developing a new generation of '-omics' profiling for the assessment of commercial GE crop plants with regard to their nutritional equivalence and food safety. Studies using *Arabidopsis thaliana* as the laboratory model plant were not examined in this paper (for further details see [6]).

GE plants with new agronomic traits, but without deliberate modifications to metabolic pathways

These 36 studies concerned crops such as barley (1 study), cabbage (1), maize (11 and [7–9]), pea (2), potato (1), rice (4 and [10–16]), soybean (2 and [17]) and wheat (3). Here we do not discuss the studies on GE lines of tomato and tobacco producing pharmaceuticals nor the use of '-omics' to identify food allergens (see [6]).

Our examination reveals that '-omics' profiling of GE varieties were similar to non-GE counterparts, although some minor differences exist between GE lines and their comparator conventional control lines. For example, the amounts of some specific metabolites were higher or lower in the GE glyphosate-tolerant soybean line [18]; these differences could be explained by modification in the regulation of the shikimate pathway. Gene expression in leaves differs more between conventional varieties than between two GE glyphosate-tolerant varieties [19]. A study on wheat found that the different genetic background of the control lines resulted in a quantitatively different flavonoid content compared to the GE fungal-resistant line whereas different GE lines showed only minor differences relative to their non-GE counterparts [20]. Natural plant-to-plant variability also exists: in a comparison of GE insect-resistant MON810 and control maize lines, some 2DE-separated protein spots showed a high variability between individual samples from the same line [7]. Some differences observed between GE lines and their counterparts can be limited to a given developmental stage as shown in a Bt (*cry1Ab/1Ac* gene) rice study [10]. In rice, herbicide-resistant Bar68-1 carrying *bar* gene and insect-resistant 2036-1a carrying *cry1Ac/sck* gene events did not substantially alter proteome profiles as compared with conventional genetic breeding and natural genetic variation [11]. Another proteomics analysis showed that these GE events (Bar68-1 carrying *bar*, *cry1Ac* and *sck* genes) in rice do not substantially alter proteome profiles as compared with conventional genetic breeding and natural genetic variation [12]. In a metabolic study of the same rice event (*cry1Ac* and *sck* genes), a slight difference in concentrations of phytosphingosine, palmitic acid, 5-hydroxy-2-octadenoic acid and three other unidentified metabolites was due to gene modification while environmental factors played a greater role than gene modification for most metabolites [13]. Proteomics analysis indicated no significant differences in Bt-rice seeds containing the *cry1ab* and *cry1/ac* genes compared to its isogenic controls [14]. Significant changes in some metabolites were found both in bacterial blight-resistant rice varieties obtained by conventional breeding or transgenesis compared to the parental non-GE variety. However, the line obtained by conventional breeding possesses a distinctive metabolite profile and shows more differences versus the parental than the transgenic line [15]. In comparison with a non-GE comparator, Bt rice showed differences in antioxidant system and signalling regulation as a response to insecticide stress [16].

Data analyses revealed variations related to factors such as variety and environment. Several environmental factors (such as field location, planting and sampling time, or crop management practices) were shown to exert a greater influence than transgenesis.

These profiling studies consistently indicate that transgenesis has fewer unintended impacts than conventional breeding. Interestingly, one study showed that transcriptome alteration was

greater in mutagenized plants than in transgenic plants [21]. Unlike transgenic lines, mutagenized lines are not subjected to food safety assessment in the EU.

None of these published assessments using new ‘-omics’ profiling points to new safety concerns about marketed GE crop varieties.

GE plants with altered metabolic traits

These 24 studies concerned GE crops such as barley (1 study), grapevine (2), maize (1), potato (5), rice (5), tomato (6 and [22]), and wheat (3).

GE lines with altered metabolic traits do not necessarily exhibit pleiotropic changes. However, some changes in compounds do occur when certain pathways are modified. As expected, several metabolism pathways for example in tomato can be altered, either in conventional mutants or in GE lines, when regulatory genes are affected [23,24].

Some differences in wheat expressing glutenin subunit genes in the endosperm are found in metabolites between GE and parental lines, but generally, they fall in the range of differences caused by environmental factors (growth in fields in different years and on different sites) [25]. Thus, larger differences were observed between two wheat parental lines than between the GE and control lines. Some changes in seed compounds of two high-Trp rice lines are found due to altered pathways which were predictable as a result of altered biosynthetic pathway but no major changes were observed for other phenolic compounds [26]. In potato, depending on genotype, somaclonal variation may be responsible for an unknown proportion of differences [27].

Long-term and multigenerational feeding studies

The inclusion of GE plants in animal feed and for direct human consumption has increased consistently since the first commercial production in 1996. However, the increased use of GE plants for human consumption and feed for livestock has led to public concern related to a perceived risk for health, toxicity and allergenicity of the transgenic proteins.

When ‘molecular, compositional, phenotypic, agronomic and other analyses have demonstrated equivalence of the GM food/feed, animal feeding trials do not add to the safety assessment’ (EFSA [28]). However, valuable information can be added to the safety assessment of GE food and feed safety by animal feeding studies, especially if doubt remains on the equivalence of GE food [2]. In these comparative feeding studies, 33% of the feed consists of GE material or control material (see recommendations of the French Agency for Food, Environmental and Occupational Health and Safety [29]); the remaining part is composed of a balanced diet. The results of 90-day rodent feeding trials performed with GE maize, rice and soybean did not lead to any unintended effects in animals (see [30]). However, we decided to address the following question: can long-term studies as well as multigenerational feeding studies detect potential unintended effects in animals (that are not detected in 90-day subchronic tests)? We examined recently 33 published studies regarding the long-term effects of GE plants, that is studies significantly longer than the 90-day tests (17 studies), as well as multigenerational studies (16 studies). These studies have been compared to the already performed 90-day studies (for further details see [30]).

We explored the issue whether GE plants may reveal any long-term effects of GE exposure not identified during the short-term premarket risk assessment.

Long-term studies

A detailed discussion on long-term studies (longer than 90–96 days) is available in [30]. Here, we update this investigation with a 16-week study on pigs fed with Bt-maize [31] (see also the short-term feeding trial [32]), a 22-week study on Japanese quail fed with Bt-maize [33], a 32-week study on Atlantic salmon [34] fed with Bt-maize and glyphosate soybean, and a 35-week study on beef cattle fed with Bt-maize [35].

All the 17 studies were financially supported by public funds. The duration of GE-based diet feeding times vary between 110 days (16 weeks on pigs fed with Bt-MON810 maize [31]) and 728 days (104 weeks on rats fed with glyphosate-tolerant (CP4-EPSPS) soybeans [30]). Rat (Fischer 344 and Wistar strains) was the predominant model (used in four studies, two in both strains). Various animal models were additionally used such as Swiss mice (five studies), salmon (three), beef cattle (one), dairy cows (one), macaques (one), pigs (one), and quail (one). Several parameters have been examined (detection of transgenic DNA, body and organ weight, blood and urine analyses, enzyme activities, biochemistry, histopathology and immunology). Most of these studies utilized major commercial products, namely glyphosate-tolerant (CP4-EPSPS) soybean (ten rodent studies along with a feeding study on salmon [34]) and insect-resistant (Cry1Ab) maize (five feeding studies on cows for 100 weeks [30], beef cattle [35], pigs [31], quail [33] and salmon [34]). In addition, one study concerned rice containing human T-cell epitope from Japanese cedar pollen allergens fed to macaques for 26 weeks [30].

Recently, a study claimed that the glyphosate-tolerant GE maize NK603 and a related herbicide formulation caused organ damage, tumors, and early death among Sprague-Dawley rats on rats fed with maize NK603 during two years [36]. However, numerous agencies of food safety, namely the German agency ‘Bundesinstitut für Risikobewertung’ [37], the European authority ‘EFSA’ [38,39], the Australian and New Zealand agency ‘Food Standards Australia and New Zealand’ [40], the Danish agency ‘Danmarks Tekniske Universitet’ [41], the Netherlands agency [42], the French agency ‘ANSES’ [43], the French High Council of Biotechnologies ‘HCB’ [44], the Belgian Biosafety Advisory Council [45], the Health Canada and Canadian Food Inspection Agency (CFIA) [46] and the Brazilian National Biosafety Technical Commission [47] refuted these claims.

A diverse range of animal models and various feeding durations and feed composition were used in these studies. No new safety concerns were raised and no supplementary information, in addition to previously performed 90-day feeding studies, were apparent. The new study carried out on pigs [32] also showed no long-term effects after 110 days (16 weeks) of feeding with maize containing Cry1Ab protein (MON810 event). Differences observed in serum biochemistry were all within the normal reference intervals for pigs; according to the authors these differences were the result of a lower enzyme-resistant starch in the GE compared to non-GE maize, which had been previously reported [31]. Changing from the non-GE maize to the GE maize diet may have resulted in a lack of satiety in pigs fed the non-GE/GE treatment.

The enzyme resistant starch content of food is known to influence satiety. The authors concluded: 'Long-term feeding of GM maize to pigs did not adversely affect growth or the selected health indicators investigated.' [32]. Previous work by the same group also found that short-term (31 days) feeding of GE maize had no adverse effects on growth [31]. No significant influence on feed intake of Bt-maize, fattening and slaughtering results were observed in a 35-week beef cattle study [35]. Feeding of Bt-maize did not impair the laying intensity and the specific and nonspecific immune response in a 22-week quail study [33] and differences in zinc serum concentrations range within the normal variation of in quail. In a 32-week salmon study, no differences were observed between Bt-MON810 and non-GE maize feed, while GE and non-GE diets resulted in higher LAP activity compared to a standard diet and activity of maltase and AcP was higher in this standard diet [34].

It is important that comparison of the GE diet is done with the non-GE isogenic counterpart [2]. The studies on maize and rice comply with these required standards to compare GE and non-GE lines. Unfortunately, six studies using a soybean-based diet do not declare whether an isogenic line was used (in five studies the event is not mentioned; see discussion in [30]).

Multigenerational studies

The main goal of these studies was to assess whether feeding a generation (n) with a GE-based diet had adverse effects on the next generation ($n + 1$). These 16 multigenerational studies were performed on animals fed with GE-based diets throughout their life or only on short-term (less than 90 days). In both cases these animals were bred to produce future generations (studies on two to ten generations) (for further details see [30,48–53]). The longest multigenerational study consisted of feeding quail with a diet containing up to 50% Bt176 maize over ten generations. The duration of GE-based diet feeding varies between 1 and 188 weeks. Rodents were predominantly used (mice in five studies (see [30,49]) and Sprague-Dawley and Wistar rats in four studies [30,52]). The farm animals used were pigs (two studies), bulls (one), dairy cows (one), goats (one), sheep (one), hens (one), and quail (one). Parameters measured included transgene detection, body and organ weight, feed intake, enzyme concentrations or activities, lactation, histopathology and hematology, reproductive factors and performance.

The GE-material in the diets utilized Bt-insect-resistant maize (in eight studies including [49–51]), glyphosate-tolerant (T25) maize [52], glyphosate-tolerant (*cp4 epsps* gene) soybean (three studies), glufosinate-ammonium-tolerant triticale (two studies), potato containing the phosphinothricin acetyltransferase (*bar* gene) and lysine-rich rice [53].

However, in two studies using a maize diet, an isogenic line was not used. The event was not mentioned in one Bt-maize study. In two studies using a maize diet and a soybean diet an insufficient number of animals was used (see discussion in [30]).

All these 16 studies were financially supported by public funds. No mutigenerational effects were reported in a majority of studies. However, effects were reported in three studies, but it should be noted that no isogenic lines were used. These differences concerned the level of LDH enzyme of target animals such as goats fed with glyphosate-tolerant soybean [54] and changes in immune responses of mice fed with glufosinate-ammonium-tolerant triticale in the

fifth generation of mice [55]. However, these differences seem to be minor, especially because the authors do not conclude that they constitute a health hazard. The authors suggest that these changes may fall within the normal range of variation but should be further investigated. It should especially be determined whether they are reproducible. An inadequate number of animals were used in a study on soybean [52]. When comparing Bt176-maize to the non-GE maize fed to sheep, some minor metabolic changes were reported with no demonstration of any health hazards [30]. The authors suggest that these changes should be further investigated to check if they are reproducible or not.

Bt-MON810 maize did not significantly influence production and reproductive performances of animals compared with a diet containing 50% isogenic maize when using pig offspring at birth [50] and pigs for 115 days postweaning [51]. No impact of glufosinate-ammonium-tolerant T25 maize on reproductive function of Wistar rats and on progeny development were found in two consecutive generations [52]. A lysin-rich rice was found as safe as its near-isogenic non-GE rice in three consecutive generations of Sprague-Dawley rats [53].

Discussion

What lessons can be drawn from the use of new 'omics' techniques on the food safety?

The 60 'omics' profiling publications comparing GE and non-GE crop varieties, with or without intentional metabolic changes, converge to show that transgene insertions produce few unintended effects [4].

Currently, the risk assessment of GE crops includes the analysis of 50–150 analytes identified by OECD consensus documents [1]. This number depends on the crop species. In the literature, metabolomics is the prevalent 'omic' approach to assess GE crops, followed by transcriptomics. To a lesser extent proteomics is also used to detect unintended effects in plants due to the genetic modification itself. Metabolic profiling of crops is becoming increasingly popular in assessing plant phenotypes and genetic diversity [56]. The use of metabolomics for regulatory GE crop assessment would be a change of paradigm (measuring more analytes, a few hundred analytes, but with less precision). Proteomics (through a 2-DE protein analysis) can be used for qualitative and quantitative estimation of the allergen levels, including new ones, with recent improvements in sensitivity, mass accuracy and fragmentation [57].

Few public laboratories have used different 'omics' approaches in a comparative approach. Therefore, an exhaustive comparative assessment of these techniques is not yet possible. These 'omics' profiling studies are highly heterogeneous (depending on plant tissues, growth parameters, range of comparators and methods). There is a need to conduct normalized, validated approaches before these techniques can be used for the routine safety of new GE crops.

Large effects due to the environment were observed in gene expression, protein, and metabolite levels in some studies, illustrating the need for exposure to the same environmental conditions, pairwise differences between GE lines and their progenitor lines. Larger differences were often observed between two conventional lines, between years of sampling, and between different field sites than between the GE and control lines. Many

methodological shortcomings are identified with ‘-omics’ approaches, a paucity of reference materials, and a lack of focused strategy for their use that currently make them not conducive for the reglementary safety assessment of GE crops [58]. For determining unintended effects in GE crop varieties, a validation work is needed before these ‘-omics’ technologies could gain full recognition by regulatory authorities and agencies.

What lessons can be drawn from the use of long-term and mutigenerational studies?

Very few published long-term feeding studies use the same animal model or the same crop model. Moreover, the parameters studied varied. Hence no studies have been carried out twice in the same conditions by different research teams. Therefore, improvements in the protocols should be made, particularly focusing on reproducibility of data.

No new safety concerns were raised in these mutigenerational studies. However, some studies suffer from weaknesses such as lack of an appropriate control group and the number of animals or the correct number of animals, lack of precision regarding duration of the study and the event studied. Statistical criticisms can also be raised: weak definition of factor levels and absence of a complete combination of factors inside experimental designs. No evaluation of the statistical power as well as few multivariate approaches were reported in these studies. Future studies should be undertaken according to EFSA recommendations which have underlined the necessity of an improved methodology when statistics are involved [59] and the distinction between statistical significance and biological relevance [60].

Conclusions

We addressed the question whether alternative techniques, such as ‘-omics’ assessments of GE plants or long-term animal feeding studies, can provide useful clues for unintended effects of GE food/feed. The application of the precautionary principle and stricter

regulations have failed to convince certain consumers that EU regulations are tough enough regarding food and feed safety. Long-term and multigenerational studies should only be conducted in a case-by-case approach for GE food/feed safety and nutritional regulatory assessment if some reasonable doubt remains after a 90-day rodent feeding trial. Thus, considering distrust in data provided by seed companies and sceptical opinion on GE crops, it is important that new approaches such as ‘-omics’ have been used by public research laboratories. However, none of these ‘-omics’ assessments have raised new safety concerns about marketed GE crop varieties. This is not surprising considering the experience acquired after 15 years of growing and consuming GE food and feed. Our review does not provide evidence that more food safety testing is necessary for GE crop varieties. These long-term and multigenerational data and ‘-omics’ data taken together suggest that, apart from specific cases, their risk assessment could be lowered.

Despite these scientific data, allegations against food safety of GE crop varieties are probably to remain in the public debate in the EU. However, it can be noticed that the French and German governments, which launched a procedure called ‘safeguard clause’ to ban cultivation of GE maize, did not use food safety arguments to justify it (in the EU a procedure called ‘safeguard clause’ allows a Member State providing valid reasons to consider that a GE crop plant constitutes a risk to human/animal health or to the environment, to provisionally restrict or prohibit the use and/or sale of that product on its territory [61]). Instead these governments tried to demonstrate environmental risks for the cultivation of GE maize, arguments which also failed to provide scientifically valid data [62].

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