



CURRENT AWARENESS
OF ISSUES RELATED TO
GENETICALLY MODIFIED FOOD
AND FOOD FROM CLONED ANIMALS

January – June 2007

Prepared as part of a New Zealand Food Safety Authority
contract for scientific services

by

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Client Report FW0771

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SUMMARY

This report is one of a series intended to provide NZFSA with information on current and emerging food safety issues related to GM Foods, and foods derived from cloned animals, which contributes to effective food policy, regulatory and risk management activities.

This report covers selected developments in the period January to June 2007, and includes:

1. Chloroplast transformation systems for transgene containment and implications for GM food safety assessment:
Chloroplast transformation has the potential to generate GM plants with multi-copy transgenes and high level gene expression. Availability of such GM crops in the future will require detailed determination of (i) their nutritional compositional equivalence, particularly of second generation GM plants, and (ii) the potential for transgene DNA to be transferred to food products derived from animals fed GM feeds
2. Cisgenics and implications for GM food legislation:
Cisgenic plants contain no genes from outside of their sexually compatible gene pool. It can be argued that cisgenic plants are therefore more similar to traditionally bred plants than are transgenic plants, and are as safe as traditionally bred plants. On this basis it has been proposed that plants derived from cisgenic gene transfer should be excluded from GMO regulations and be treated the same as plants bred using traditional or mutational breeding systems.
3. Intragenic vectors and implications for GM food legislation and testing:
The intragenic vector system is an extension of the cisgenic gene transfer system. It can be argued that the genetic rearrangements that result from intragenic vector transformation of plants are no different to those seen using traditional translocation and mutation breeding and so should not be required to comply with regulations governing the growing and processing of GM crops. As all the DNA in a plant transformed using an intragenic vector system is derived from that plant species, or a sexually compatible relative, it becomes harder to test for GM plants. There are no generic tests currently available that would detect such transformation events.
4. Are GM and conventionally bred cereals really different?
Using advances in –omics and analytical chemistry techniques it was shown that GM and non-GM wheat were substantially equivalent. This supports previous studies on compositional equivalence of GM crops to non-GM counterparts, however, does not suggest that compositional equivalence analysis should not be undertaken on a case-by-case basis.
5. Regulation of foods derived from animals fed GM feeds.
Information provided supports the contention that food products derived from animals fed GM plants are no more likely to present dietary risk than food product from animals feed with conventional plants.

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1 INTRODUCTION

This project is intended to provide the New Zealand Food Safety Authority with an independent source of current information on genetically modified foods (GMFs) and foods from cloned animals. The principal activity of this project is to survey the current scientific literature to keep abreast of developments in key areas of food safety, selecting five key articles within the subject areas specified and providing comment on the significance to NZFSA for use in its policy, regulatory and risk management activities.

- The studies/topics have been chosen from within the following subject areas:
- Novel techniques for developing GM plants/animals and the implications on current detection methods;
- Animal feeding studies – specifically within the area of foods derived from GMFs or foods from cloned animals;
- Food safety and/or composition studies on GMFs and/or foods from cloned animals;

Adventitious presence issues for GMFs and new GM varieties approved for food use, with particular emphasis on describing how other countries have responded with regard to audits and/or testing regimes and safety assessments, and providing relevant information and discussion of the actual food safety risks.

This is the second report for the 2006/2007 year and covers the period from January to June 2007.

Wider issues concerned with environmental or social effects of genetic modification and genetically modified organisms (GMOs), biodiversity, gene transfer, insect resistance, etc., are not covered in this report. This reflects the division of responsibility for genetically modified material, between the New Zealand Food Safety Authority and Food Standards Australia New Zealand (FSANZ) for GMFs on one hand, and the Environmental Risk Management Authority (ERMA) for GMOs on the other.

For consistency, some alternative terms have been standardised in this report. “Corn” and “maize” are interchangeable; in this document “corn” is used throughout. Canola is a genetic variation of rapeseed (or oilseed rape) developed by traditional plant breeding to be low in both erucic acid and glucosinolates (“double low” variety). In this document “canola” is used for this “double low” variety of rapeseed.

Abbreviations used throughout this document:

EU: European Union

FSANZ: Food Standards Australia New Zealand

EFSA: European Food Safety Authority

USDA: United States Department of Agriculture

USFDA: United States Food and Drug Administration

2 NOVEL TECHNIQUES FOR DEVELOPING GM PLANTS

2.1 CHLOROPLAST TRANSFORMATION

GM crops currently approved by FSANZ all contain a novel gene construct inserted into the nuclear genome of the plant. The resultant GM plants are selected to contain single-copy insertions that are inherited in a Mendelian fashion. Containment of GM plants is an ongoing concern that relates to the potential for GM plants to out-cross with non-GM plants, either of the same crop species, such as with adjacent field plots, or with wild relatives.

In plants nuclear genes are bi-parentally inherited, that is, from both the paternal (pollen) and the maternal parent. Plant cells, however, also contain organelles that have their own genetic material, specifically the mitochondria and plastids. Organelle genes are known to be predominantly maternally inherited. Plants with transgenic chloroplast (a type of plastid) genomes (transplastomic plants) offer an alternative to the established nuclear transgenic plants. There is the potential to ‘contain’ the transgene by excluding it from the pollen of the plant. Transplastomic plants also offer technological advantages such as (i) the ability to establish high-level transgene expression, (ii) a convenient platform to develop transgene pyramiding, and (iii) an avoidance of issues associated with epigenetic¹ instability of nuclear genes.

The methodology to transform chloroplasts with ‘foreign’ DNA was established in the 1990s, and the field of chloroplast genetic engineering is now entering the phase of field testing and commercial development. How strict maternal inheritance is, however, has not been fully established. During this reporting period two publications were released by the prestigious journal *Proceedings of the National Academy of Sciences (USA)* that detailed studies to better determine the level of maternal inheritance of organelle genetic material and the implications of this for genetic engineering of plants. A background to chloroplast inheritance and transformation is provided, along with a summary of results from these two studies.

2.1.1 Background to chloroplast inheritance and transformation

Chloroplasts are complex, discrete green structures, or organelles, contained in the cytoplasm of plant cells. Chloroplasts give the green colour of almost all plants and the only plants without chloroplasts are those that do not make their own food, such as non-green parasitic plants. Within the chloroplast is a system by which plants capture the energy from sunlight and convert it into food – the photosynthetic machinery. Chlorophyll, a molecule involved in photosynthesis, is responsible for the green colour of chloroplasts. Similar organelles which don’t contain chlorophyll are also found in plant cells and these are generically referred to as plastids. Mitochondria, the other energy producing organelle in eukaryotic cells, are similar to chloroplasts.

As early as the late 1800s it was observed that the green colour in plants was one of the easiest traits to follow in plant breeding experiments. Although some pigmentation traits were inherited as expected with Mendelian inheritance, other colour traits were seen to only

¹ heritable traits over rounds of cell division, and sometimes transgenerationally, that do not involve changes to the underlying DNA sequence

pass to progeny through the female (maternal) parent that provides the cytoplasm of the embryo. These observations led to the hypothesis that chloroplasts must carry their own genes. By the 1960s it had been demonstrated that organelles contain their own DNA and the necessary genetic machinery to enable gene expression.

Chloroplast genomes are circular DNA molecules and range in size from 120-160 kilobases. A single chloroplast can contain between 30 and 200 copies of this genome, and a single mature photosynthetic leaf cell contains on average 50 chloroplasts. This equates to up to 500,000 chloroplasts and up to 1 million copies of the chloroplast genome per square cm of photosynthetic leaf tissue. While these figures are variable depending on the plant species, and a range of environmental and physiological factors, the figures do indicate the potential for introduction of a transgene at a very high copy number per plant cell if introduced into a chloroplast. This has advantages if the transgene is one that it is desirable to have expressed at a high level in the plant cells.

Chloroplast inheritance is maternal in most flowering plants and is achieved by several different mechanisms. In some plant species all the plastids are distributed into vegetative cells during pollen development, so that the generative cells that form the pollen are plastid free. In other species young paternal generative cells may acquire a few plastids but these are excluded from the embryo during fertilization.

Methods to insert transgenes into the genomes of plastids were developed in the 1980s. These methods rely on direct insertion of transgenes as *Agrobacterium*-mediated transformation only inserts genetic material into the nuclear genome of plants. Current methods for chloroplast transformation include biolistics, polyethylene glycol (PEG)-mediated transformation and a novel microinjection approach.

Biolistics is the most efficient of these methods and involves penetrating the plant tissue with tungsten or gold particles coated with DNA. Prior to the development of biolistic transformation methods chloroplast transformation was considered to be virtually impossible because the chloroplast is enclosed in a double membrane that is a physical barrier and there are no viruses or bacteria known to infect chloroplasts that could be used as a vector for gene transfer. With biolistics, tungsten or gold particles coated with DNA are shot through the chloroplast envelope by a helium powered gun. In a successful transformation event the DNA is deposited in a chloroplast and becomes integrated into the chloroplast genome. The major limitations of this method are that it is expensive to perform and the chosen plant tissue must be able to be efficiently regenerated in culture from a single cell. Chloroplast transformation also requires the development of specific transformation constructs that contain chloroplast DNA elements flanking the transgene cassette. This is necessary as the novel DNA introduced into the chloroplast can only be incorporated into the chloroplast genome by a homologous recombination mechanism that recognises chloroplast sequences. Insertion of a transgene into one chloroplast genome generates a situation known as heteroplasmy. By subjecting the transformed tissue to selection pressure for the inserted gene, such as using targeted antibiotics, it is possible to retain the insertion over subsequent chloroplast and cell replications until a homoplasmic system is obtained where all of the chloroplast genomes in a cell/plant have the insert (see Fig 1)

Figure 1: Transformation of chloroplast by biolistic method.

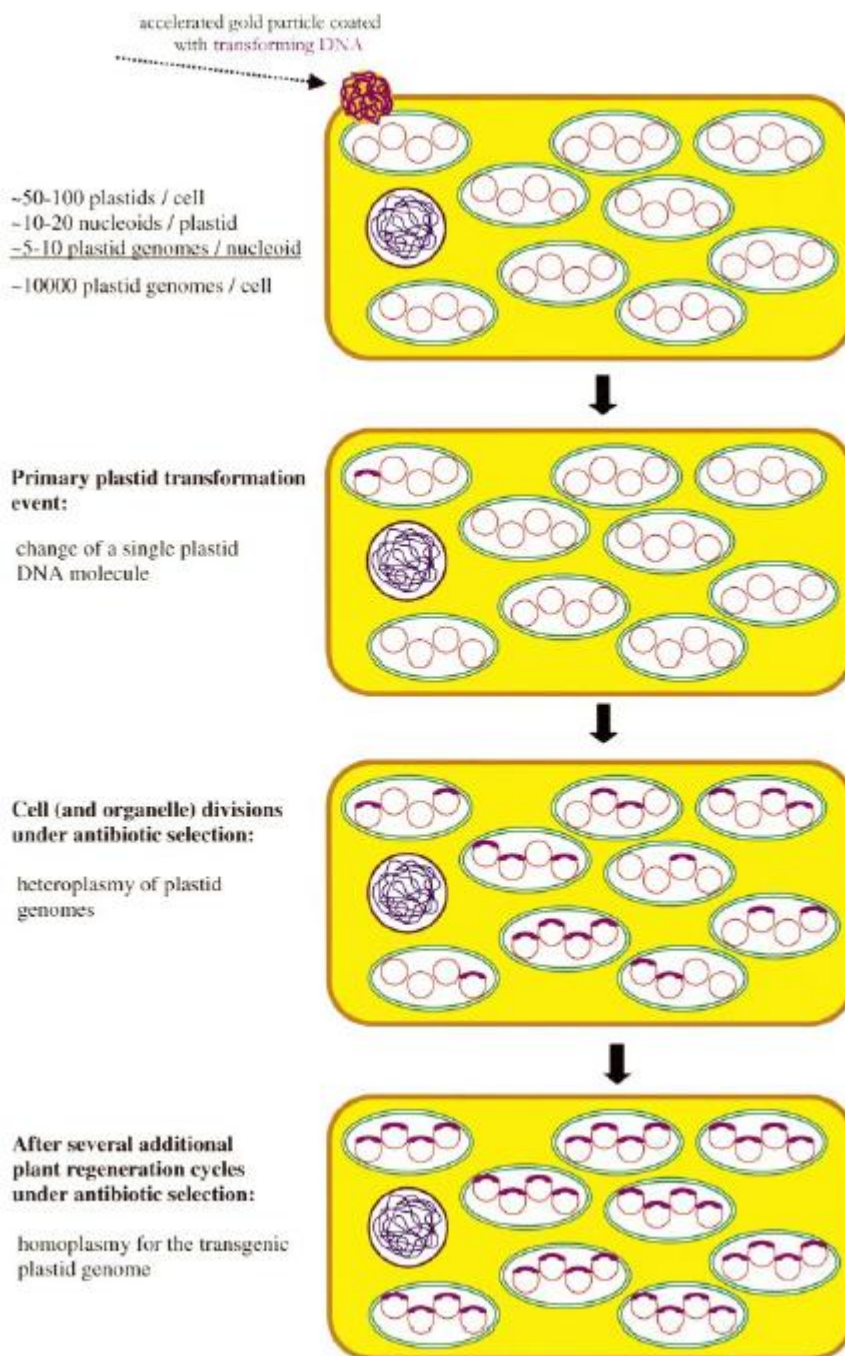


Diagram from:

http://www.ag.usask.ca/departments/plsc/undergrad/classes/plsc416/projects_2002/christine/webpage/transf.html

Accessed on: 14 August 2007

Transformation of plastid genomes was advocated as a means of containing gene flow from GM plants, by prevention of transgene incorporation into the pollen of the plant. However, following initial trial transformations it was reported that there was a relatively high frequency of paternal DNA transmission of transgenes in plant species in which the plastids

were previously considered to be solely maternally inherited, including tobacco and petunia. This has serious implications for the effectiveness of this technology for transgene containment. The issue of paternal transmission of transgenes inserted into plastids was addressed by two groups of researchers and reported in the April 2007 issue of PNAS.

2.1.2 Assessment of paternal transmission of transplastomic DNA integrations

Both research groups used tobacco (*Nicotiana* sp) as the transformation system. Currently tobacco is the only plant species in which plastid transformation is a routine technique and current data for chloroplast inheritance in tobacco is contradictory. The researchers utilized different experimental systems to assess the inheritance of transplastomic genes in tobacco.

Ruf *et al.* (2007), from Max-Planck-Institute in Germany, developed a transplastomic tobacco line that contained an introduced antibiotic resistance gene and a gene for a green fluorescent protein (GFP) able to be directly visualised in plant tissue using a fluorescent microscope. This line was used as the paternal (pollen donor) plant in a cross with a male sterile female parent. Antibiotic selection pressure was used to establish homoplasmic transgenic lines and these were assessed for expression of the *gfp* gene. Experimental methodology also took into account the possibility of point mutations in the chloroplast genome that could confound the results. Of the 2.1 million seedlings screened 39 lines (0.0018%) were confirmed as containing transgenic chloroplasts. Most of these lines had transgenic chloroplasts only in their cotyledon tissue and these chloroplasts were rapidly lost along with the cotyledons. Only six seedling lines (0.0002%) contained stable paternally-derived transgenic chloroplasts in apical meristem tissue – the tissue from which seedling developmental growth occurs. This equated to a frequency of paternal transmission of chloroplasts of $\sim 2.86 \times 10^{-6}$. The researchers argue that because tobacco has a strong tendency for self fertilization (<10% progeny resulting from cross-fertilization events), along with a requirement for a separation corridor of 10m between GM and non-GM crops, this frequency of transmission would be even lower in a field situation and would be more likely in the range of 10^{-8} . That is, one tobacco plant with paternally-derived chloroplasts per 100 million fertilization events.

Swab and Malign (2007) tested paternal chloroplast transmission in tobacco using a different approach. The paternal (pollen donor) tobacco line carried an antibiotic resistance gene in its chloroplast genome. Seedling lines that had obtained their chloroplasts from the paternal parent would therefore be expected to be antibiotic resistant. Three progeny with paternally-derived chloroplasts from a total of 34,115 seedlings (0.008%) were recovered, a frequency of 9×10^{-5} . A maternal tobacco line was also developed that contained a nuclear genome from one tobacco species and a cytoplasm (including organelle genomes) from a different tobacco species. This alloplasmic maternal line was male sterile due to a trait carried on its mitochondria. Seedling lines from a cross with this maternal parent that had obtained their chloroplasts from the paternal parent would be expected to be antibiotic resistant and male sterile. Ten seedlings with paternally-derived chloroplasts from 47,859 seedlings (0.02%) were obtained, a frequency of 2×10^{-4} . Of the 12 total paternal plastid transfer events only two showed paternal plastids in the apical meristem tissue of seedlings. This equates to 0.002% stable paternal plastid transfer, a frequency of 2 seedlings with paternal plastids for every 100,000 seedlings.

The experimental approach taken by Swab and Malign (2007) also enabled them to test for the intactness of paternally derived plastid DNA and for co-transmission of paternal plastid

DNA and mitochondrial DNA. It was determined that the entire paternal plastid genome was transferred from the pollen parent, rather than small plastid fragments. There was no evidence for transfer of plastid DNA to the nuclear genome, or of recombination of plastids by fusion. From these results it was concluded that the plastid DNA is likely to be inherited as entire organelles. Only paternal plastid DNA was detected in seedlings acquiring their plastids from the pollen parent, however, it was shown that each of the seedling lines that had paternally-derived plastids had bi-parental mitochondrial DNA. That is, seedlings acquired both maternal and paternal mitochondria. This indicates a system of co-transmission of organelles and is the first report of this phenomenon.

2.1.3 Implications of chloroplast transformation for GM crops

Both publications reported pollen transmission of chloroplasts in tobacco as rare events, in the range of 0.0087-0.00024%. Current FSANZ regulations require labelling of products if they contain 1% or more of a specific ingredient as GM. Chloroplastic transformation systems would therefore be likely to provide much lower levels of adventitious field contamination of pure crop products (eg: corn kernels) than is currently permitted. It will however, be necessary to determine background levels of paternal chloroplast transmission for individual crops as rates are likely to vary between plant species, and even within cultivars of a species.

The low levels of pollen transmission of chloroplasts detected in these studies, compared to higher levels previously reported result from the experimental methods used in these studies which (i) removed the confounding issue of point mutations that can cause antibiotic selection anomalies, and (ii) determined the location of transgene expression so discounting any transfer events that were not retained in the apical meristem tissue of seedlings.

Chloroplastic engineered GM plants are currently beginning field trials, for example, a transplastomic tobacco engineered to express human interferon for pharmaceutical use. Several agronomic traits, including insect resistance, herbicide resistance, disease resistance, drought tolerance and salt tolerance have been engineered into chloroplast genomes. Crops in which chloroplast genomes have been able to be transformed include soybean, tomato, potato and lettuce. High level gene expression has been demonstrated in transplastomic GM plants – giving up to 46% of the total soluble protein in the plant.

As crops engineered by chloroplast transformation become available commercially there will be a need to monitor the impact of multi-copy transgenes and high level gene expression on:

- i) nutritional compositional equivalence of GM food products, particularly those from second generation GM plants (see Section 2.2.1.1), and
- ii) the potential for transgene DNA to be transferred to food products derived from animals fed GM feeds (see Section 2.2.1.2).

Sources:

Ruf, S., Karcher, D. and Bock, R. (2007). Determining the transgene containment level provided by chloroplast transformation. *PNAS (USA)* 104(17) 6998-7002.

Svab, Z. and Maliga, P. (2007). Exceptional transmission of plastids and mitochondria from the transplastomic pollen parent and its impact on transgene containment. *PNAS (USA)* 104(17): 7003-7008.

Additional information from:

Danielle, H. (2007) Transgene containment by maternal inheritance: Effective or elusive? *PNAS (USA)* 104(17): 6879-6880.

2.2 CISGENIC GENE TRANSFER SYSTEMS

Cisgenic plants (or intragenic plants) are described as those plants engineered with genes derived from the plant itself or from a close sexually compatible relative. The argument has been made that because these plants contain engineered genes from within the 'natural' gene pool of the recipient plant they should be exempt from the legislative requirement for GM plants. Evert Jacobsen and Henk Schouten from the Wageningen University in the Netherlands published a review of cisgenic gene transfer and implications for regulations in *Trends in Biotechnology* in May 2007. A summary of information presented in this review is provided.

Classical plant breeding provides two main ways for genetic improvement of plants – crossing and mutation. Developments in biotechnology over the past thirty years or so have resulted in methods to improve plants by genetic modification/engineering. Genetically modified plants (GM plants) were initially developed using systems that introduced genes from widely different organisms into the plant. For example, the methodology relied on the use of antibiotic resistance introduced via a bacterial gene to enable selection for successful gene transfer events. Generically referred to as transgenesis, this technology has enabled the extension of the traditional gene pool of a plant species. The novel genes transferred into plants can provide traits that do not occur in the recipient plant in nature, nor are they able to be introduced into the recipient plant by traditional breeding techniques. Regulations put in place regarding the growing and use of GM plants have been largely driven by concerns related to the perceived risks of cross-species gene transfer. Food safety issues relate to the safety of a 'foreign' gene in a food plant and its potential impact on composition and

nutritional adequacy and safety issues like potential allergenicity and toxicity. Jacobsen and Schouten argue that cisgenic plants more closely reflect the situation seen for induced translocation and mutation breeding and as such should be regulated under similar legislation.

2.2.1 Classical and modern plant breeding practices

Plant breeding has always had the aim of improving the domesticated performance of a plant species and has targeted such traits as plant size, plant vigour, increased seed output, improved resistance to disease and improved tolerance to abiotic stress. Many of these traits require a degree of genetic variation greater than is found within the gene pool of the recipient plant. Traditional breeding systems have been developed to introduce desirable traits into plants from other sexually compatible species. One of the major limitations of this out- or wide-crossing is that the desirable trait may be introduced into the recipient plant along with a large amount of other non-desirable genetic material from the donor species. This non-desirable material may compromise agronomically important traits in the recipient plant. For, example out-crossing of a domesticated potato line with a wild relative in order to improve disease resistance may compromise tuber size.

Introgression breeding is a system of interspecific hybridization between a recipient plant and a donor plant that involves a number of subsequent backcrosses with the recipient line, coupled with selection for the introduced trait. This aims to reduce the amount of donor genetic material in the recipient plant not associated with the desirable trait. Over a number of generations the introduced trait can be introgressed into the recipient plant. This type of plant breeding however, often results in ‘linkage drag’ from the donor plant. Linkage drag occurs when genes encoding undesirable traits are closely linked to the desirable genes within the genome of the donor plant. Even over a number of back-crossing generations it is not possible to physically separate these regions. The retention of one region in the recipient plant will always be accompanied by the retention of the other region. The degree of linkage drag in a particular introgression breeding system will depend on the degree of genetic recombination that can occur around the target gene, the number of backcross generations and the ease of selection against genes with negative side effects. While some new molecular technologies such as marker assisted selection can reduce linkage drag problems they are still frequently encountered in crops with large, complex genomes and crops that are vegetatively propagated.

Induced translocation breeding begins the same way as introgression breeding. After a number of backcrosses and selection for the desirable trait seeds from the resulting line(s) are irradiated with gamma-radiation. Following more selection in subsequent generations plants can be found that contain the target desirable gene translocated in the recipient genome with a minimum of the remaining donor chromosome material linked to the target gene. However, such events are rare and the insertion of the donor target gene is random – that is, different lines from the irradiated seed can contain the gene translocated on different chromosomes. In this situation any persisting linkage drag cannot be easily removed by additional backcrossing.

2.2.2 Mutation breeding

Mutations are perhaps the ultimate source of genetic variation, however, their usefulness in plant breeding is limited by the recessive nature of most mutations at gene level and that, by definition, mutations are single cell events. Mutations therefore need to be established as homozygous traits before they can be usefully employed in plant breeding. This is possible with seed propagated crops by repeated crossing, however, it is not possible in heterozygous, vegetatively propagated crops. Many seed propagated crops with homozygous parents have been improved by establishment of induced mutations, for example, barley, rice, tomato, and corn. Various types of mutation are possible. They may be intragenic, intergenic or genome changes such as polyploidy. At the level of DNA sequence changes can include base substitutions, frameshifts, inversions, deletions and duplications, translocations and insertions by natural transposons. Mutations result in changes to the genetic material of the plant and the exact positioning of these changes in the genome are not always discernable. Mutation breeding can be effected by the use of gamma irradiation or mutation inducing chemicals such as EMS (ethyl methyl sulphonamide). These treatments often result in additional damage to chromosomal DNA during the mutation induction, followed by cellular repair. The integrity of this repair is not always exact and can result in undesirable effects in the mutated line.

2.2.3 Genetic modification for plant breeding

Transgenesis

During the early development of plant genetic engineering, transgenes that originated from non-crossable species were the main gene source. For example, antibiotic resistance genes from bacteria that were used as selectable markers to detect successful transfer events. Systems were developed to introduce novel gene constructs into plants either utilizing the gene transfer mechanism from the natural soil-borne bacteria *Agrobacterium sp.*, or by direct transfer mechanism such as biolistics (see Section 2.1.1.1). The choice of transgene depended on the desired trait and the availability of *in vitro* genetic material from which to develop transformation cassettes. Initially GM plant lines were developed for such traits as herbicide tolerance and insect resistance, utilizing transgenes from bacteria and insects respectively.

Cisgenesis

Cisgenesis is the introduction into plants of isolated genes, along with their native promoters, from the target plant itself or from a crossable relative. This application of genetic engineering of crop plants has been enabled by recent developments in the design of marker-free transformation vectors, and vectors that enable marker genes to be removed from the engineered plant after the experimental phase of plant selection (see Report FW07007 Section 2.1.1 for a discussion of cre/lox technologies for marker gene excision). Advances in the sequencing of genomes from a range of crop plants have also provided information on gene sequences that can be utilized in cisgenic engineering. Cisgenic transformation allows breeders to access genetic traits such as disease resistance genes that may be found in wild relative, without the associated problems of linkage drag that are encountered with traditional introgression or induced translocation breeding systems. Cisgenics also enables the breeder to stack resistance genes from different sources, such as from the same chromosomal position

on different species or accessions of a crop. An example of where cisgenics could be usefully applied would be in the development of apple scab resistance in cultivated apples. The *Vf* gene from a wild apple relative is known to confer resistance to the disease, however, fifty years worth of traditional breeding programmes have been unable to introduce this gene into cultivated apples without negative effects on fruit taste and texture. This is due to linkage drag. Using cisgenics the *Vf* gene should be able to be introduced into cultivated apples in a much shorter time period without these associated non-desirable effects.

2.2.4 Comparison of breeding methods and implications for regulation

The testing and release of genetically modified plants is tightly regulated internationally to prevent any negative effects on either the environment or human health. Most regulations relate to the potential for transgenesis to extend the gene pool of the recipient plant species. Regulatory bodies and lawmakers have concentrated on risks associated with ‘novel’ genes in plants in terms of the biosafety framework established to control these risks. Jacobsen and Schouten (2007) contend that cisgenic plants are more aligned with those generated by translocation and mutation breeding than they are with transgenics and so should be exempt from the regulation governing GM plants. The justifications for this are summarised below:

1. In cisgenic plants the gene of interest and its promoter is present in the intended recipient species or in a crossable relative. Therefore the gene pool of the recipient plant species is not being extended. Cisgenics therefore carries no more risk to the environment than traditional breeding and has no risk of introducing unintended allergenicity or toxicity into a food crop.
2. In cisgenics the donor gene is inserted into the recipient plant at an unknown position in the genome. This is analogous to trans-gene insertion and the position of the insertion can have effects on the expression of the gene, even if it is under the control of a cis-promoter. The insertion results in a ‘mutation’ of the recipient plants genome sequence at the insertion site, and rearrangements or translocations may occur in the flanking regions. The argument can therefore be made that insertion of the cis-gene is not consistent with the situation of chromosome re-shuffling that underpins sexual reproduction. A biological counterargument to this can be made as translocations and alteration to systems that regulate gene expression (e.g. demethylation) have been shown to occur in nature. A regulatory counterargument is that induced translocation breeding also results in translocation of DNA fragments to unknown insertion sites and mutational breeding results in large changes to the plant’s DNA sequence. Neither of these breeding systems is currently regulated under GMO legislation.
3. In sexual reproduction an allelic sequence of one parent is replaced in the progeny. In cisgenics a sequence is added to the species genome. As a consequence of the transfer methodology it is also possible that the sequence may be added in more than one position, which may affect gene expression and therefore phenotype. However, gene duplication and multigene families is a common natural occurrence in plants, with resistance phenotypes as an example. Increase in the ploidy levels of a plant is also a common example of gene duplication, is widely found in traditional plant breeding and is not regulated under GMO legislation.

4. Cisgenic plants might contain small, non-coding sequences derived from transfer vectors, such as the T-DNA borders that are required to effect the transfer of DNA into the plant's genome. Such sequences are generally very small, for example the T-DNA border sequences are only 25 base pairs long. The argument is made that these small vector-derived sequences are non-coding and therefore unlikely to have any phenotypic effect. Recently researchers have identified DNA sequences within plants that can function as border sequences, raising the possibility of gene transfer systems that contain no trans-elements (see Section 2.1.2 on intragenic vectors).

In summary Jacobsen and Schouten (2007) maintain that, while cisgenics is a form of genetic modification of plants, because cisgenic plants contain no genes from outside of their sexually compatible gene pool they are fundamentally different from transgenic plants. They argue that cisgenic plants are more similar to traditionally bred plants and are as safe as traditionally bred plants. Therefore, plants derived from cisgenic gene transfer should be excluded from GMO regulations and be treated the same as plants bred using traditional or mutational breeding systems. Given the potential for cisgenesis to speed up the breeding process in plants, particularly the ability to obtain durable multigenic resistance phenotypes, they suggest avoidance of such legislative requirements would greatly enhance the economical and environmental prospects of agriculture.

To date no cisgenic plant has been put forward for authorisation under GM food legislation so this remains a subject for debate. Such debate can be found in a number of responses to Jacobsen and Schouten's suggestion in *Nature Biotechnology* (Various November, 2006).

Source:

Jacobsen, E. and Schouten, H.J. (2007). Cisgenesis strongly improves introgression breeding and induced translocation in plants. *Trends in Biotechnology* 25(5): 219-223.

Additional information from:

Schouten, H.J., Krens, F.A. and Jacobsen, E. (2006). Cisgenic plants are similar to traditionally bred plants. *EMBO reports* 7(8): 750-753.

Various. (2006). 'Cisgenics' as a product designation. Letters to the Editor, *Nature Biotechnology* 24(11): 1327-1333.

2.3 INTRAGENIC VECTOR GENE TRANSFER SYSTEMS

The development of GM plants over the past 20 years has offered advances to agriculture and horticulture by extending the gene pool of genetic material from which to introduce novel traits into plants. As discussed in the preceding section on cisgenic gene transfer, the initial research on gene transfer into plants utilized transgenes from across species barriers. While providing a number of GM crops with advantage to the producer, the introduction of 'foreign' DNA into plants has raised concerns with the general public. Ethical concerns such as "playing God" and safety concerns related to introduction of transgenes into food products

have affected public acceptance of GM crops and has driven regulatory requirements for their production.

Over recent years the expansion of 'genomic' technologies has increased the knowledge of plant genes and genomes. Annotated genome sequences of all major crop plants are likely to be available within the near future. To date a complete genome sequence is available for rice, and genome sequencing projects in progress include wheat, corn, sorghum, potato, tomato, soybean, cassava, banana, grapevine and *Brassica oleracea* (which includes cabbage, cauliflower and broccoli). This improved knowledge of plant genomes means that more genes are being identified from plant species that would benefit their sexually related agricultural counterparts. The potential to transfer these genes directly into related plant genotypes and the benefits of this over traditional breeding systems is discussed in the preceding section on cisgenic gene transfer systems.

Intragenic vectors, designed to transfer DNA into plants, contain only DNA derived from the genome of the recipient plant species or that of a sexually compatible relative. Construction of intragenic vectors for gene transfer has been proposed as a mechanism for developing cisgenic plants that contain no cross-species DNA. As discussed in Section 2.1.2.4, cisgenic plants engineered using cisgenes in a traditional transformation vector still contain small fragments of cross-species DNA as a part of the vector used to effect the transfer. Assoc. Prof. Anthony (Tony) Conner of Crop & Food Research, Lincoln, has been developing a system by which transfer vectors can be constructed that only contain DNA sequences from within the recipient plant's natural gene pool. In early 2007 Conner and co-workers published a review of progress towards developing these intragenic vectors. Background to the gene transfer system, a summary of Conner's work, and implications for GM plant regulations and testing is provided.

2.3.1 Requirements for vector-based DNA transfer into plants

Gene transfer into plants can be effected by two general methods:

- (i) *Agrobacterium*-mediated gene transfer, and
- (ii) direct DNA transfer.

Agrobacterium-mediated transfer makes use of the ability of the naturally occurring soil-borne plant pathogen *Agrobacterium sp.* to insert DNA into the genome of a recipient plant. Through this mechanism *Agrobacterium* strains are capable of inducing crown gall or hairy root tumours on plants by transferring a section of DNA (the transfer, or T-DNA) containing genes that induce tumour formation and enable biosynthesis of two derivatives of the amino acid arginine; ornithine and nopaline. The T-DNA is found in *Agrobacterium sp.* on the Ti or Ri plasmids, along with other genetic elements (the virulence, or *vir* genes) required for the gene transfer process. The combined action of the *vir* genes and several other chromosomal based genes in *Agrobacterium sp.* results in the transfer and integration of the T-DNA into the nuclear genome of plants. This system can be considered as a sophisticated form of parasitism.

During the development of plant genetic engineering as a method of plant improvement it was determined that the *vir* genes and the T-DNA region could be separated in

Agrobacterium onto two different plasmids, and the *vir* gene products could still act in *trans* to effect transfer of the T-DNA (see Fig 2 & 3).

It was also found that the only requirement of the T-DNA for transfer is the presence of delimiting short, imperfect direct repeats of about 25bp, known as the left and right border sequences. The genes within the native *Agrobacterium* Ti and Ri plasmid T-DNA regions that resulted in tumour formation could be deleted to overcome their negative effect on the ability to regenerate plants in cell culture. The region between the left and right border sequences could be replaced with a gene construct(s) for a desirable gene and, so long as the *vir* functions were provided in *trans*, this construct could be integrated into the genome of the target plant. This led to the development of a range of binary vectors for plant transformation. A generic binary vector system is shown in Fig 4.

The T-DNA transfer initiates from the right border sequence, and is very precise, with only 3-4 nucleotides of the right border being transferred to the plant. The end point of the T-DNA transferred at the left border is less precise and may occur at or near the left border, or even well beyond it. For this reason it is possible to get left border truncations, and conversely vector backbone sequences can integrate into the plant genome at relatively high frequency. While there are a number of selection strategies to select against vector backbone insertion events, they do not necessarily identify all partial backbone sequence integrations. Such insertions are one of the major issues raised in safety assessment of GM crops as they have the potential to facilitate unintended effects in the plant – such as encoding unintentional open reading frames for novel proteins.

Ongoing modifications to binary vector systems has resulted in the development of ‘minimal T-DNA vectors’ for use in agriculture. These vectors contain the minimum elements required for efficient T-DNA transfer. They are very small T-DNA sequences with a selectable marker gene tightly inserted between the left and right border sequences and a short region with a series of unique restriction sites for inserting genes-of-interest. It has also been shown that deletion of the left border sequence has minimal effect on T-DNA transfer, however, removal of the right border sequence abolishes transfer. It is still advantageous to retain two borders flanking the T-DNA, however, as it helps to define the initiation and end points of transfer and facilitates the recovery of transformation events without additional vector backbone sequences.

Figure 2: Native *Agrobacterium* Ti plasmid and gene transfer event

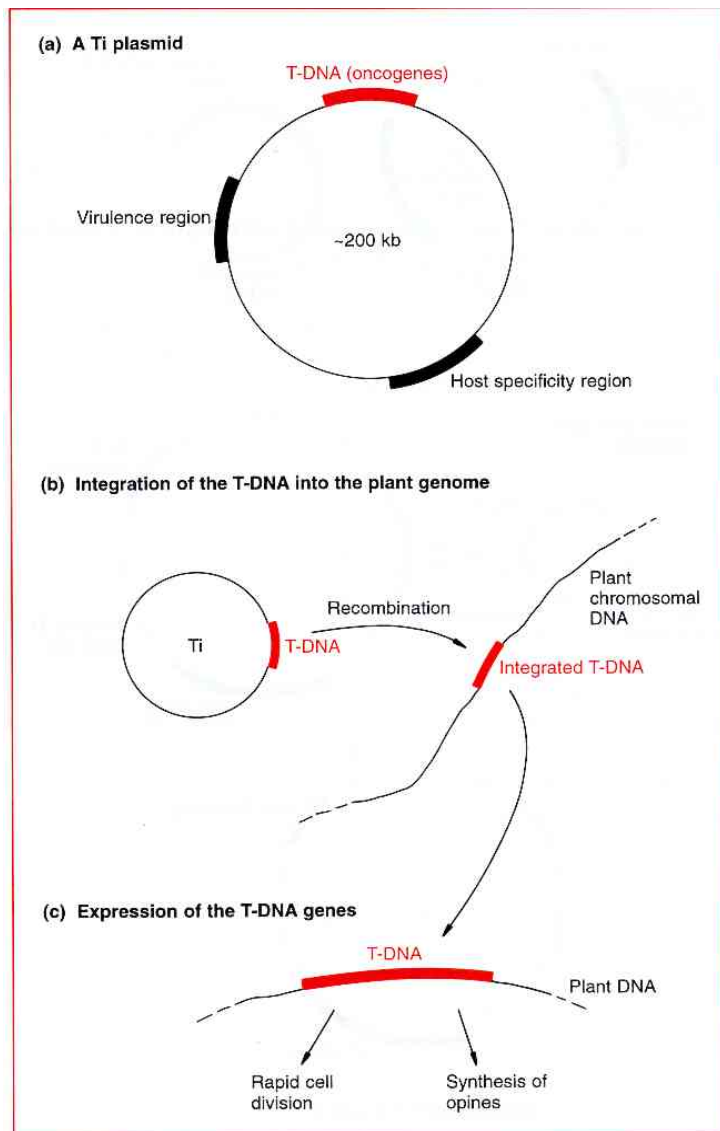


Figure 3: Separation of Ti functions onto two plasmids

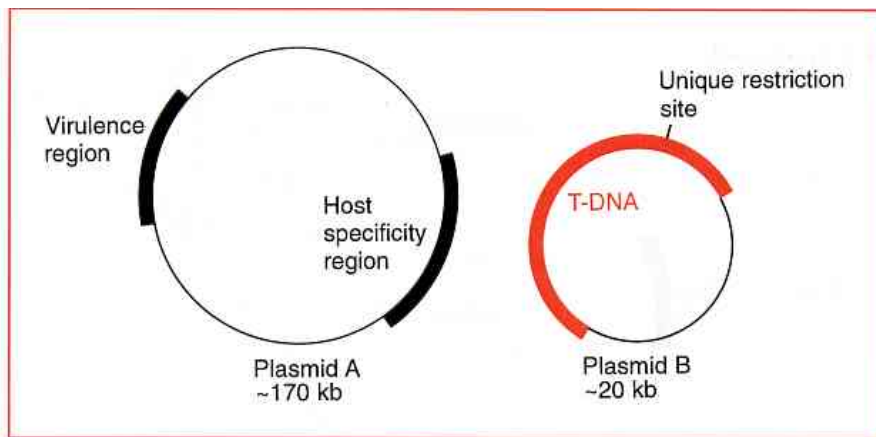
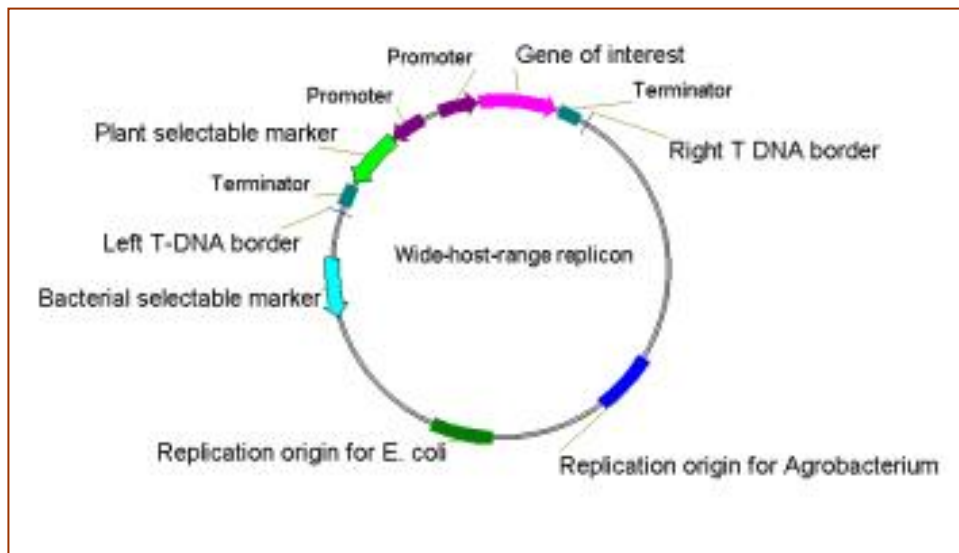


Figure 4: Generic binary transformation vector



2.3.2 The intragenic vector system

The intragenic vector system is an extension of the ‘minimal T-DNA vector’ system. The system aims to develop T-DNA vectors capable of efficient gene transfer to a host plant species, but using vectors that consist of only DNA that originates from the host species, or related species to which it is able to naturally hybridise. To develop such a system requires identification of the functional equivalents of vector components from within plant genomes and then using these sequences to assemble vectors for plant transformation.

The minimal components needed for an effective plant transformation vector were identified as:

1. A plant-derived T-DNA-like region that should contain two (or at least one) T-DNA border-like sequence (s) in the correct orientation, and a series of restriction sites for cloning the gene(s)-of-interest.
2. An origin of replication.
3. A selectable marker (usually an antibiotic resistance gene).

Items two and three are required to maintain the vector in *E.coli* for laboratory manipulation and in *Agrobacterium sp* for the transformation event. A fully intragenic vector would also contain a cisgenic gene(s)-of-interest. Several different approaches have been undertaken to develop intragenic vectors that meet these requirements.

The P-DNA approach

P-DNA is an acronym for plant DNA. The method involves replacing the entire *Agrobacterium* derived T-DNA sequence with a plant-derived transfer DNA (P-DNA). A PCR method was used to screen a pooled sample from 66 potato accessions for DNA fragments flanked by regions with homology to *Agrobacterium* border sequences. A 391 base pair fragment flanked by sequences with sufficient homology to the border sequences was obtained. Proof of principle was demonstrated by inserting a plant-expressed gene for resistance to the antibiotic kanamycin between the borders and placing the P-DNA plus gene-of-interest cassette on a traditional binary vector backbone. The P-DNA region was shown to be effective for *Agrobacterium*-mediated transformation of potato.

The limitations to this approach are that, while shown to be effective for potato, the general presence of such P-DNA fragments within the genomes of other crop plants has not been determined. The method requires the presence of a DNA fragment within a genome that has a number of specific requirements; (i) preferably two T-DNA border-like sequences orientated as direct repeats, (ii) a separation of less than 2 kilobases between these border-like sequences, and (iii) an intervening span of sequence with appropriate restriction enzyme sites suitable for cloning the gene intended for transfer. The probability of finding such feature on a relatively short fragment within a plant genome is likely to be small.

Assembly of plant-derived T-DNA-like regions

An alternative approach to the P-DNA approach is to join together two or more DNA fragments from the same plant species to construct a T-DNA-like region. The shorter sequence motifs of border-like sequences are likely to occur at much higher frequency in a chosen plant genome than the longer P-DNA sequence. Plant genomes can be searched for DNA sequences containing these motifs and plant-derived T-DNA-like regions can be assembled by joining these together. Vectors with plant-derived T-DNA have been assembled *in silico* from a range of plant species, including tomato, potato, apple, rice and onion. Proof of concept has been demonstrated by constructing a plant-derived T-DNA-like region from petunia, inserting it into a standard binary vector backbone and testing it using marker-free *Agrobacterium*-mediated transformation. Analysis using PCR showed the plant-derived T-DNA-like region was able to effectively transfer to petunia.

Chimeric right T-DNA borders

Creation of a chimeric right T-DNA border is a more specific approach to generating plant-derived T-DNA-like regions. During the transfer of the T-DNA to the plant the right T-DNA border is 'nicked' such that only the 3-4 nucleotides immediately preceding the T-DNA region are transferred to the plant genome. For an intragenic vector, therefore, only these 3-4 nucleotides need to be of plant origin, while the remaining right border sequence can be derived from *Agrobacterium sp*. This approach has been successfully demonstrated in transformation of *Arabidopsis thaliana*.

Whole plant-derived vectors

To fully eliminate any foreign DNA contained in a binary vector it is possible to construct a vector entirely from plant DNA-derived sequences. Transformed plants generated using these vectors contain no foreign DNA even if vector backbone sequences are transferred to the plant. Plasmids derived entirely from plant sequences should also be able to be used to generate intragenic transformed plants using direct DNA transfer systems such as biolistics.

As noted above, along with the T-DNA region a binary vector needs to include an origin of replication and a selectable marker to maintain the vector in bacterial systems. Functional equivalents to these components have been identified from plant genomes and can be assembled by joining together two or more fragments from the same species.

Considerations for the design of intragenic vectors

There are a number of considerations identified as important when designing intragenic vectors from homology searching of plant genome sequences:

1. The DNA fragments making up the T-DNA-like region should preferably not contain known regulatory elements, such as promoters, as such elements may have unintended influences on expression of the target gene-of-interest.
2. The DNA fragment on which the T-DNA is based should not be derived from heterochromatic regions (non-coding, non-expressed, condensed DNA), as this may negatively influence expression of the gene-of interest.

These two limitations can be overcome by selecting genome sequences from databases of ESTs (expressed sequence tags), which are DNA sequences known to be transcribed in the plant. Currently most plant genome sequence data is of EST origin.

3. For intragenic transformation it is beneficial to include up to 2 kilobases of known plant DNA sequence in the vector sequence outside the T-DNA left border region. This allows for imperfect transfer of the left border, without compromising the cisgenic nature of the transformation. When a vector is completely derived from plant DNA sequence the additional vector elements such as origin of replication can serve as this additional DNA.
4. The incorporation of selectable marker genes in a plant-derived T-DNA-like region is often not necessary with easy to transform crops like potato. Analysis by PCR can be used to identify plants containing the transferred DNA. However, for crops that have low efficiency of transformation a plant selectable marker may be desirable. Intragenic marker genes have been identified in plant genomes, such as mutant forms of endogenous genes capable of conferring resistance to specific herbicides. For example, over expression of the endogenous *A.thaliana Atwbc19* ABC transporter gene confers kanamycin resistance.
5. The intragenic T-DNA region designed for transfer into the recipient genome should be composed of as small a number of DNA fragments as possible. This allows the transferred region to mimics the natural DNA rearrangements that can be seen to occur in plants.

2.3.3 Implications of intragenic vectors systems

Intragenic vector systems have implications for both regulation and testing of GM plants. The intragenic vector system is an extension of the cisgenic gene transfer system discussed in Section 2.1.2 and presents challenges to the regulation of GM plants in the same way. It can be argued that the genetic rearrangements that result from intragenic vector transformation of plants are no different to those seen using traditional translocation and mutation breeding. As such, plants generated by intragenic vector transformation could be considered to lie outside of the current definitions of GM plants and so not be required to comply with regulations governing the growing and processing of GM crops.

As all the DNA in a plant transformed using an intragenic vector system is derived from that plant species, or a sexually compatible relative, it becomes harder to test for GM plants. There are no generic tests currently available that would detect such transformation events. The only way to definitively show a transfer of genetic material has occurred is to determine the presence of expected junction sequences at the insertion sites. This requires the provision of detailed sequence data on the vector construct from the construct developer and would require specific tests for each transformation construct. The implications of this on long-term monitoring of intragenic transformants has logistical and cost implications for testing bodies.

With the development of cisgenics and intragenic vector transfer systems, a continuum now exists for crop improvement from traditional breeding practices through to genetic manipulation that inserts foreign gene sequences into plants. Defining a clear point of demarcation on which to base a legal definition of genetic modification, which is biologically relevant and enforceable becomes very difficult. This is likely to provide ongoing challenges for lawmakers and regulatory monitoring agencies.

Source:

Conner, A.J., Barrell, P.J., Baldwin, S.J., Lokerse, A.S., Cooper, P.A., Erasmuson, A.K., Nap, J-P and Jacobs, J.M.E. (2007). Intragenic vectors for gene transfer without foreign DNA. *Euphytica* 154: 341-353.

3 FOOD SAFETY AND COMPOSITION STUDIES

3.1 ARE GM AND CONVENTIONALLY BRED CEREALS REALLY DIFFERENT?

To date the GM crops that are most predominant in the food market are the so called “1st generation” GM plants. These plants have been engineered with “input” traits that benefit the grower, such as herbicide and pest resistance. While agronomic performance and crop yield remain important key targets in engineering food crops there is an increasing emphasis on development of GM plants with “2nd generation” output traits. These are traits that benefit the processor and/or consumer. Examples of these output traits for cereal crops are improved dough properties for bread-making performance of wheat, increased vitamins and minerals, reduced glycaemic index and increased content of soluble fibre. Because these 2nd generation

modifications will affect the composition of the major staple food crops there is likely to be renewed debate on the safety and acceptability of GM food crops.

Risk assessment for GM foods currently requires a demonstration of nutritional adequacy and compositional equivalence. This equivalence is most often assessed by analysis of crude nutrients, fibre constituents, amino and fatty acid profiles and minerals, as well as for the presence of any undesirable substances (e.g. mycotoxins). Recently it has been suggested by a number of ‘watch-dog’ organisations that GM foods risk assessments should include a wider range of analyses to determine changes to profiles of gene expression products, proteins and metabolites.

Researchers at the Rothamsted Research Centre in the UK have undertaken a series of comparisons of conventionally bred and 2nd generation GM cereals and have utilized a suite of –omics as well as analytical chemistry methods to assess the compositional equivalence of the crops. Results from this research were published in *Trends in Food Science & Technology* early in 2007. A summary of the methods employed and the overall results obtained is provided.

3.1.1 Technologies for comparing GM and conventionally bred crops

As wheat and most other major cereal crops have been consumed by humans for many years the main concept used to evaluate the safety of modified forms of cereals is that of substantial equivalence. A lack of rigorous definition of this concept has, however, made it useful for industry but often unacceptable to consumers. For the purposes of this study the researchers have defined substantial equivalence to mean that the composition of the GM food is within the range of variation shown by conventionally bred cultivars, when grown under similar conditions.

The levels of analysis used to determine substantial equivalence are summarised in Fig 5. At the genomic and transcriptional level the methods used are designed to confirm that transgenes behave similarly to endogenous genes with respect to their stability and inheritance over several generations. The effect of transgene expression on the total profile of gene expression products in the plant is also analysed. New methodologies that employ microarray systems can result in high throughput analysis. For example, the commercially available Affymetrix array system for wheat can detect the expression of about 55,000 genes, approximately half of the total number of genes present in the genome of wheat. Determination of gene expression profiles is considered to be important as it is currently not possible to control the site of transgene insertion and the final positioning within the recipient plant genome could have an influence on expression of the transgene, expression of endogenous genes and/or generation of novel unintended proteins. This latter issue is also addressed by comparison of the protein profile of the GM plant with a conventional counterpart. While it is obvious that the GM plant should differ to a conventional counterpart in expression of the transgene, there should be no “knock on” effects on the expression of other genes.

Although comparison of gene expression patterns is important there is no certainty that any differences which are identified between a GM plant and a conventional counterpart at this level will result in any difference in the protein profile of the plants and that any differences

seen during plant development will be reflected in grain composition and properties at harvest. Proteomic analysis, most often using two dimensional gel electrophoresis, can compare protein profiles of plants. While useful, this methodology is laborious to perform and often unreliable for making comparisons between results from different experiments or experiments performed by different laboratories. Protein array systems have been developed in the research environment but are not readily available for routine analysis. In contrast to proteomics, metabolomic analysis, to profile the composition of low molecular mass metabolites like sugars and amino acids, can be performed in high through-put automated systems using methods such as mass spectrometry. The analysis of GM plants compared to conventional counterparts should also take into account the equivalence of functional properties and the stability of these from year to year and across environments.

3.1.2 Comparative analysis of 2nd generation GM wheat with conventional counterparts

3.1.2.1 Production of transgenic wheat line

The HMW (high molecular weight) subunits of gluten make up about 10-12% of the total gluten proteins in wheat. Gluten composition of wheat has a direct effect on dough strength and a higher number of HMW subunit genes in a wheat genome are directly correlated with improved dough strength in processing. Addition of extra gene copies of HMW subunits is a method that has been employed to engineer wheat with improved grain quality for processing.

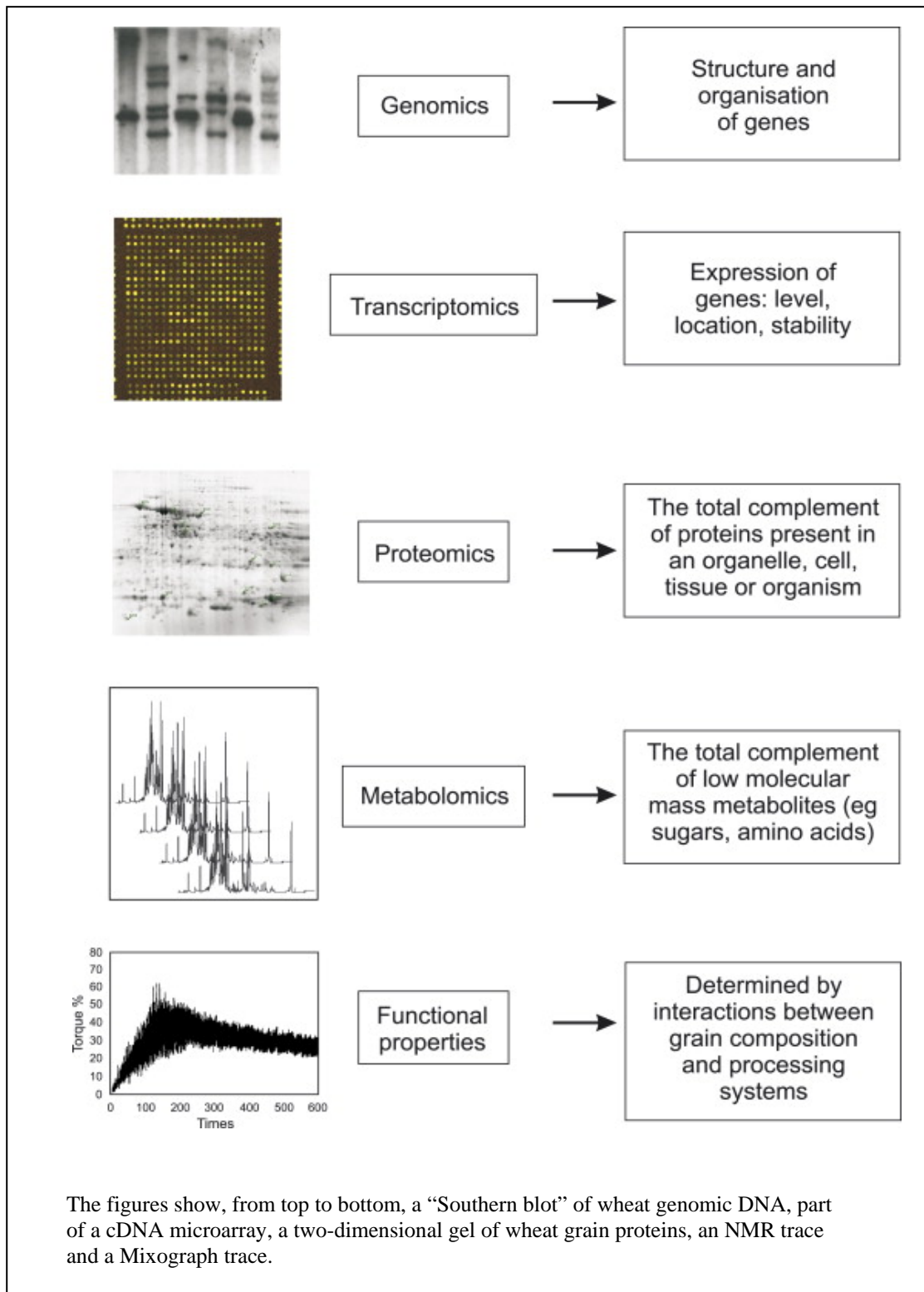
The researchers at Rothamsted engineered several lines of wheat with transgenes encoding HMW gluten subunits. The HMW genes were expressed under the control of their own promoters, limiting expression to the developing endosperm of the grain. Marker genes included in the transformation were for the *bar* gene (which confers resistance to the herbicide Basta), and the *uidA* gene (which encodes the B-glucuronidase enzyme and enables identification of transformants via a colourmetric reaction). These two marker genes were under the control of a constitutive corn promoter, resulting in ubiquitous expression in all plant parts at all times. Transgenic wheat lines were produced that contained ‘clean fragments’; that is, T-DNA insertions that had no transformation backbone vector sequences co-transferred. In addition, transgenic lines were selected that contained additional plasmid DNA from the transformation vector backbone. These non-T-DNA insertions are of concern to regulatory agencies and risk assessments for GM foods require evidence of their absence from transformed crop lines.

3.1.2.2 Transcriptome comparison of glasshouse grown lines

Transcriptome comparison (the profiling of expressed gene sequences) was carried out on a limited number of transgenic lines and appropriate controls that were grown under glasshouse conditions. The small scale of this analysis was because transcriptome analysis is expensive and time consuming. Six lines were grown in three glasshouse experiments. Analysis was carried out on developing endosperm tissue in grain shortly after fertilization and in young leaves. Transgenic lines were compared to parental lines for the transformation, siblings derived from the same cross as the parental line and to a commercial wheat cultivar. The overall pattern of gene expression showed that there were a small number of differentially

expressed genes between transgenic lines and the parental control, while a large number of genes were differentially expressed when the parental line was compared to siblings from the cross from which it was derived. This result was seen to be consistent with the contention that transgenesis is a very precise and controlled method of crop improvement compared to conventional breeding, where many thousands of genes may differ between lines generated by the same cross.

Figure 5: Summary of analysis used to define substantial equivalence of GM crops



Taken from Shewry *et al.* (2007)

The function of a selection of differentially expressed genes was predicted based on function of characterised genes in other crop species. No data is actually provided in the paper, however, the authors' claim that none of the differentially expressed genes identified in this study gave obvious cause for concern in relation to human health or field release.

3.1.2.3 Comparison of field grown lines

Environmental conditions can have a major impact on grain composition and end use properties. When developing elite cereal lines, either by conventional breeding or by genetic engineering, it is therefore important that the lines be stable despite year-to-year and site-to-site variation in environmental conditions. To assess the long-term stability of the 2nd generation transgenic wheat lines a series of replicate field experiments were carried out over four years. These trials were carried out on two sites within the UK with different environmental conditions. Comparisons were made to the same control lines as described above, grown under the same conditions. A range of measurements was taken including dry weight, nitrogen content, determination of gluten protein composition, metabolite profiles and dough mixing properties. The major discriminator between results was found to be year, and in some cases site, with no significant distinction between transgenic and control lines. The only exceptions were for expected changes in the transgenic lines related directly to the introduced transgene. Ongoing proteomic comparisons are being undertaken to identify protein changes which were not related to the expression of the HMW subunit transgenes and associated marker genes. Changes to the metabolite profile of transgenic populations were identified as involving the levels of maltose and sucrose, and of the common amino acids aspartic acid and glutamine. None of these differences are considered to pose a risk to human health, and the differences did not exceed the overall range of variation related to site and year.

Overall the study has allowed the researchers to draw several important conclusions about the substantial equivalence of GM and non-GM wheat. That is, that:

1. The expression of transgenes in the lines studied is not intrinsically more or less stable than that of the corresponding endogenous genes.
2. The transgenic and control lines show similar stability in agronomic performance and grain functional properties when grown at multiple sites over several years.
3. The gene expression profiles in developing grains of transgenic and control lines are much more similar to those of the parental lines than are the profiles of lines produced by conventional plant breeding
4. The metabolic profiles of control and transgenic lines usually fall within the range of variation which is observed between genotypes of the species or samples of the same genotype grown under varying environmental conditions.

These conclusions have been drawn based on a more extensive suite of tests than is usually required for current risk assessment of GM crops, but affirms what is reported in most risk assessments. This does not mean that all GM crop are necessarily substantially equivalent to their non-GM counterparts but does support the contention that it is possible to engineer elite 2nd generation wheat crops by transgenesis that are substantially equivalent to non-GM wheat, within what is able to be tested for using advances in -omics and analytical chemistry techniques.

Source:

Shewry, P.R., Baudo, M., Lovegrove, A., Powers, S., Napier, J.A., Ward, J.L., Baker, J.M. and Beale, M.H. (2007). Are GM and conventionally bred cereals really different? *Trends in Food Science & Technology* 18:201-209.

3.2 GENETICALLY MODIFIED FEED DOES NOT AFFECT MEAT

Currently there are no regulations internationally that require food derived from animals fed GM material to be labelled as such. This issue is, however, one of general public concern and such labelling could be seen to offer the consumer an informed choice. The EU Regulation (EC) No 1829/2003 on GM food and feed foresees labelling requirements. The European Commission approached the EFSA in March 2007 with a request that they provide a report on the potential for transgenes or their products to be incorporated into animal tissues or animal derived food products such as milk and eggs. This request was in response to a petition to label food products from animals that have been fed with GM feed. The EFSA subsequently released a statement based on a literature review of studies on the fate of recombinant DNA from GM feed. The review relied heavily on reference to the work of Flachowsky *et al.* (2007). Summaries of Flachowsky's paper, and the report by the EFSA, are provided.

3.2.1 Studies on feeds from genetically modified plants (GMP) – Contributions to nutritional and safety assessment. Flachowsky, G., Aulrich, K., Böhme, H. and Halle, I. (2007). Animal Feed Science and Technology 133: 2-30.

This paper summarises studies undertaken since 1997 at the Federal Agricultural Research Centre (FAL), Germany. A total of eighteen studies with feeds from genetically modified plants (GMPs) in animal nutrition are reported. Animal species included dairy cows, growing bulls, growing and finishing pigs, laying hens, chickens for finishing, and growing and laying quails. Most experiments (16) were undertaken with first-generation GMPs, that is, plants with input traits and without substantial changes in composition. These included Bt-corn, Bt-potatoes, Glufosinate tolerant (Pat)-corn, Pat-sugar beet, and Roundup Ready™ soybeans. Two studies were undertaken with so-called second-generation GMPs. These are plants with output traits, or substantial changes in the composition of a nutritional constituent. These included rapeseed with an altered fatty acid profile and inulin producing potatoes.

The selected feeds were analysed for their composition (crude nutrients, fibre constituents, amino and fatty acid profiles and minerals), as well as for the presence of any undesirable substances (e.g. mycotoxins, alkaloids, glucosinolates, lignin and phytate). Comparison was made with isogenic, non-GM lines. Most of the feeds were grown alongside their isogenic counterparts in fields managed by the Research Centre. Bt-potato and Roundup Ready™ soybean were provided by other institutions.

Animal studies were then carried out to determine the nutritional impact of the feeds, by assessment of digestibility, feed intake, health and performance of target species, as well as the effect on the quality of food derived from the target animal. Reproductive issues were considered in studies with quails and laying hens. The fate of DNA, especially transgenic DNA, during feed processing and following digestion by the animals was also assessed.

3.2.1.1 First-generation GMPs

A summary of the feeding trials undertaken with first-generation GMPs is shown in Table 1.

Table 1: Animal feeding trials with 1st generation GMPs

Genetically modified plant	Animal feed trial
Bt-corn (silage for ruminants; grain for pigs and poultry)	Beef cattle Sheep Growing and finishing pigs Laying hens Broiler hens Growing and laying quails
Bt-potatoes	Broiler chickens
Pat-corn	Pigs
Pat-sugar beet (including top silage)	Sheep Pigs
Roundup Ready™ soybean	Pigs

The results obtained supported the conclusion that the first-generation GMPs tested showed no significant difference in composition or in their physiological effect as feed for the farm animals tested when compared to isogenic non-GM counterparts. This is in agreement with other studies that show substantial equivalence between feeds from transgenic plants and their isogenic counterparts.

3.2.1.2 Second-generation GMPs

A second-generation GMP is characterized by:

- Increased content of desirable substances (e.g. amino acids, fatty acids, vitamins, enzymes etc), and/or
- Decreased content of undesirable substances (e.g. mycotoxins, alkaloids, glucosinolates, lignin and phytate).

Due to the variable nature of intended composition of 2nd generation GMPs no standard test procedures are currently available to investigate their equivalence as animal feeds. Different experimental designs are necessary to accommodate the specific nature of the transgenic event. Particular consideration needs to be included in experimental design to accommodate any potentially negative side-effects that may occur. This study reports two feeding trials with 2nd-generation GMPs:

1. A rapeseed line with increased content of the fatty acids myristic and palmitic was assessed as a feed for pigs.
Except for the intended increase in the fatty acids, the GM rapeseed showed only marginal differences in the composition of other nutrients compared to an isogenic non-GM counterpart. However, the glucosinolate content of the transgenic line was increased. While the digestibility and energy content of the feed was comparative to

the non-GM counterpart, pigs fed the GMP showed a lower daily feed intake and therefore daily weight gain. This was attributed to the increased content of undesirable glucosinolates in the GMP and demonstrates the need to perform a complete compositional and nutritional assessment on GMPs; particularly 2nd-generation, output trait, GMPs.

2. Potatoes developed to synthesize high molecular weight fructan as inulin were fed to pigs. Inulins are oligosaccharides produced by a range of plants as storage molecules. Plants storing inulins do not generally store carbohydrate as other polymers such as starch. Inulins are classified as soluble fibre and are not digested by enzymes in the small intestine of humans. This has led to their development as a food 'additive', with low caloric value, to replace the carbohydrate and fat content of processed foods. Inulins are, however, metabolized by gut microflora in the lower intestine and positively modify the profile of beneficial microflora. This feature has led to their increasing use as a pre-biotic component of functional foods in human nutrition; and this was the intended use of the transgenic potato line.

The nutrient composition of the GM potatoes was not significantly different to that of a non-GM isogenic line, except that the starch content of the GM line decreased as carbohydrate was stored as inulin. The overall carbohydrate storage capacity of the GM line was not affected by the genetic modification, and this result simply reflects the switch in carbohydrate polymer stored. However, the total alkaloid content of the transgenic potato tubers was about 25% higher than that of the non-GM counterpart. This increase in the level of an undesirable compound in a 2nd-generation GM line, concomitant with the intended nutritional change, parallels that seen for the fatty acid-modified rapeseed line discussed above. As expected with a feed containing inulin, there was a lower energetic feeding value obtained with the GM potato line, and the average daily live-weight gain of pigs fed transgenic silage was lower than that of pigs fed the non-transgenic counterpart. It should be noted that there was a limited amount of the transgenic feed available, which limited the number of animals able to be fed in the trial. This had implications for the robustness of statistical data generated and differences were not able to be confirmed at the $p > 0.05$ level.

The results of these feeding trials with 2nd-generation GMPs confirms the importance of rigorous compositional and nutritional analysis of GMPs in order to confirm that unfavourable effects may not occur along with the intended genetic modification.

3.2.1.3 Fate of transgenic DNA

The fate of DNA during processing and feeding of GM plants was assessed in a number of studies.

1. During feed processing

Transgenic *pat*-rapeseed (resistant to the herbicide phosphenthrin) was treated under four industrial processing conditions and compared with an isogenic non-transgenic counterpart using PCR methodology. The study showed that mechanical treatment had no effect on the stability of DNA, while the processes of extraction and toasting (desolventizing) caused a high degree of degradation (fragmentation) of the DNA for both lines. The size of the DNA

fragments retained after processing were all smaller than the functional gene sequence assayed for.

The effect of the ensiling process was studied with pat-corn. Both corn cob mix and whole plant silage made from the transgenic corn line were compared with an isogenic, non-GM counterpart using the same PCR methodology as above. Both types of processing resulted in DNA fragmentation in both corn lines. As with the industrial processing, the fragments remaining after treatment were all smaller than the functional gene sequence assayed for.

2. After ingestion by animals

The fate of DNA from GMPs after ingestion was studied in dairy cows, beef cattle, growing-finishing pigs, laying hens, broilers and quails fed Bt-corn, Bt-potatoes, Roundup Ready™ soybeans or inulin potatoes. A summary of the main results is shown in Table 2.

Table 2: Fate of DNA from plants after ingestion by animals*

DNA source	Animal species	Results Detection of “foreign” transgenic DNA	Detection of “foreign” non- transgenic plant DNA
Bt-corn, grain and silage	Broilers, Layers, Fattening Cattle, Dairy Cows	No transgenic DNA in animal tissues	Plant DNA fragments in muscle, liver, spleen, kidneys of broilers and layers, not in blood, muscle, liver, spleen, kidneys of finishing bulls, in eggs and feces of broilers and layers and in feces of dairy cows
Bt-corn, grain	Pigs	Transgenic DNA fragments up to 48 h in the rectum, not in blood, organs and tissues	Plant DNA fragments in the gastrointestinal tract, in blood, organs and tissues
	Broilers	Transgenic DNA in the gastrointestinal tract. No transgenic DNA in blood, organs and tissues	Plant DNA fragments in the gastrointestinal tract, in blood, organs and tissues
	Quails (10 generations)	Transgenic DNA-fragments (211 bp) in the stomach and whole gastrointestinal tract. No transgenic DNA fragments in muscle, liver, stomach, spleen, kidney, heart and eggs	Plant DNA fragments in the gastrointestinal tract
Bt-potato	Broilers	No transgenic DNA in muscle, liver, kidney and spleen	Plant DNA fragments in muscle, liver, kidney and spleen until 8 h after feeding
Roundup Ready™ soybeans, fullfat seeds	Pigs	No transgenic DNA in muscle, liver, kidney and spleen	Plant DNA fragments in the gastrointestinal tract
Inulin potato, silage	Pigs	Transgenic DNA-fragments (104 bp) in the stomach, no transgenic DNA-fragments in animal tissues	Plant DNA fragments in the gastrointestinal tract, no plant DNA fragments in animal tissues

* Table adapted from Table 27 Studies of the transfer of “foreign” DNA fragments into farm animals. Flachowsky *et al.* (2007).

These results show that while most ingested plant DNA (transgenic or non-transgenic in origin) is degraded in the gastrointestinal tract some fragments have been found in animal tissues. However, in these studies no fragments of transgenic DNA were detected in any tissue or organ sample, including eggs and milk obtained from animals fed GMPs. As some

plant DNA seems to be able to be absorbed into the animal's system from ingested feed it can't be ruled out that transgenic fragments may not also be absorbed. Based on results from this study the potential frequency of this occurring is likely to be extremely low.

Conclusions drawn by this publication and supported by the results of studies outlined include:

- GMPs without genetic modifications to specifically alter their nutritional composition (so-called 1st-generation GMPs) do not differ significantly in their nutritional value from those of isogenic non-GM counterparts grown in similar conditions.
- Strategies for nutritional and safety assessment which are developed for 1st-generation GMPs cannot be directly applied to GMPs with intended changes in nutritional/compositional constituents (2nd-generation GMPs). Assessment studies of these plants need to be designed on an individual basis to accommodate likely intended and possible unintended changes to the composition of the plants.
- To date no fragments of transgenic DNA have been detected in any organ or tissue derived from animals feed GMPs. Based on evidence for the presence of non-transgenic plant DNA in animal tissues, such an event cannot be completely ruled out, however, the likelihood is very low. DNA fragments of plant origin that have been detected in animal tissues are small in length and unlikely to be genetically functional.

3.2.2 EFSA statement on the fate of recombinant DNA or proteins in meat, milk and eggs from animals fed with GM feed

<http://www.efsa.europa.eu>

The EFSA statement is based on a literature survey undertaken at the request of the European Commission. Several aspects of the fate of recombinant DNA and protein were considered and are summarised below.

1. The fate of recombinant DNA and protein during feed processing and ensilaging.
The work of Flachowsky *et al.* (2007) is cited, along with that of other researchers who have shown that while mechanical treatments have no influence on the stability of DNA, the further processes of extraction and toasting result in highly fragmented (degraded) DNA. Ensilaging of forage crops also causes degradation of the DNA. No comment is made on the fate of recombinant proteins during processing or ensilaging.
2. The fate of recombinant DNA and proteins in the gastrointestinal tract of livestock.
Comment is made that, in principal, all feed (and food) contains DNA and proteins and that the gastrointestinal tract of animals and humans has developed in a situation of exposure to these compounds. Chemically the DNA introduced into transgenic crops, and the resulting proteins, are equivalent to those from existing foods that have always been eaten. DNA and proteins are released from ingested plant material by normal digestive processes. The mechanical process of chewing, along with enzymes in the saliva of the mouth begin the process of degradation of these chemicals. This is then followed by acid hydrolysis and enzymatic breakdown in the stomach and intestinal tract. DNA is digested into small fragments and individual nucleotides

while proteins are broken down into peptide units and individual amino acids. These breakdown products are either absorbed as a part of normal nutritional uptake or are excreted.

3. The survival of recombinant plant DNA in the human gastrointestinal tract.

Specific mention is made in the statement of studies on the fate of recombinant DNA and protein in the gastrointestinal tract of humans. This work was undertaken in 2004 and involved human volunteers; twelve healthy and seven who had undergone ileostomies. Volunteers were fed a meal containing GM soy. The transgene DNA did not survive passage through the intact gastrointestinal tract of healthy volunteers. The amount of transgene DNA surviving passage through the small intestine was determined from the volunteers with ileostomies. There was evidence for low-frequency recovery of transgene DNA, up to a maximum of 3.7% recovered in one individual. There was no evidence of gene transfer from the GM soy to the microflora of the small bowel in volunteers during this trial, although there was the possibility of some transfer in individuals prior to these experiments. While no comment on the likely reason for this is made in the EFSA statement it would be reasonable to assume that volunteers may have been exposed to GM soy in their normal daily eating and low level transfer of GM DNA to the gut microflora may have occurred over a non-distinguishable but possibly lengthy time period.

4. The potential absorption of the digested fragments of DNA or protein into animal tissues.

As outlined above, the fate of most ingested DNA and proteins is to be broken down by normal digestive processes. These processes are likely to minimise the opportunity for absorption of intact DNA or proteins. Reference is made to a number of studies designed to determine whether recombinant DNA and proteins can be detected in products and tissues from animals fed GM material. To date there is no report of specific recombinant material being detected. In one instance small fragments of the recombinant *cry1a(b)* and *cpesp* genes were found in conventional milk samples. However, these sequences were also detected in organic milk samples and it is suggested that they resulted from fecal or airborne contamination of the milk, or from the natural environment via the soil bacteria *Bacillus thuringiensis* and *Agrobacterium sp.*, from which these sequences originally derived. There are also reports of fragments of natural multi-copy plant genes (e.g. those from chloroplasts) that have been found in some animal tissues and fluids. This would suggest that uptake of fragments of DNA or protein from the intestinal tract into the body is a normal physiological process. The lack of evidence for transgene DNA or protein uptake is likely due to (i) the low chance that recombinant material stays intact long enough in the gastrointestinal tract for absorption to occur, along with (ii) transgenes currently being present in GM plants at low, single-copy-gene levels, making potential absorption a rare event and difficult to detect. Should GM plants be developed that contain higher copy number transgenes (e.g. via organelle transformation, see Section 2.1.1) the potential to detect absorbed DNA or protein fragments may increase.

5. The potential for biological functionality of absorbed DNA and protein fragments.

For ingested DNA or proteins to be biologically functional in the host organism they first need to overcome the previously discussed hurdles of remaining intact through the gastrointestinal system and being absorbed into the organism. Following absorption there are then further issues that need to be overcome. These include:

- Avoidance of endogenous DNA and protein degradation systems (largely enzymatic) designed to eliminate ‘foreign’ DNA and proteins from cellular systems.
- Integration of the ‘foreign’ DNA into the host genome of a eukaryote is essential for its ongoing ability to be expressed. The probability of such integration is low as the primary mechanism for incorporation of genetic material into the genome is via homologous recombination, which selects against ‘foreign’ sequences.
- Integration into the genome must also allow for transcription of the ‘foreign’ DNA to occur. This requires insertion of a full expression system, or the sequence must insert into a transcriptionally active region of the genome in the correct orientation and adjacent to the necessary expression promoter and terminator regions.

The low probability of any of the above occurring suggests the likelihood of functionality of ingested DNA is extremely small.

To date there is no evidence that any plant derived proteins are expressed in tissues of animals that have consumed the plant material. No plant gene or fragment of a plant gene has been detected in the human genome, or that of any other animal species tested. Where multi-copy plant DNA was seen in certain animal tissue (see above) no evidence was seen for plant gene sequences endogenously in the animal genome. Hence the DNA in the tissue was not derived from integrated genomic DNA in the animals. Studies in mice have shown that ingestion of unique recombinant DNA over eight generations resulted in no functional gene expression or germ-line transfer of the orally ingested DNA.

The overall conclusions drawn by the EFSA, based on the reviewed literature were:

1. *Biologically active genes and proteins are common constituents of food and feed in varying amounts. After ingestion, a rapid degradation into short DNA or peptide fragments is observed in the gastrointestinal tract of animals and humans.*
2. *To date, a large number of studies with livestock have shown that recombinant DNA fragments or proteins derived from GM plants have not been detected in tissues, fluids or edible products from farm animals like broilers, cattle, pigs or quails.*

3.2.3 Implications for labelling

The large number of studies summarised above support the contention that food products derived from animals fed GM plants are no more likely to present dietary risk than food product from animals fed with conventional plants. While it is acknowledged that

legislation for the labelling of food products derived from animals fed GM material would provide the consumer with a degree of personal choice, the inability to monitor such labelling would preclude its usefulness. The current lack of detection of any recombinant DNA or protein in any animal tissues tested would make the development of monitoring assays for specific animal products problematic. Compliance monitoring would have to rely on producer voluntary compliance, with no ability to survey food products.