



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

July – December 2006

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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 July to December 2006, and includes:

1. Use of *Cre-lox* systems for generating marker-free GM crops.
2. Use of -omics technologies in the assessment of food safety of GM crops.
3. Methodologies to assess the allergenic potential of food from GM crops.
4. Adventitious contamination of US rice exports with unapproved line LLRICE601 and the response of the global market.
5. Release of documents by the USFDA assessing the food consumption risk of cloned animals and their progeny.

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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 GM or cloning;
- GM and cloning food safety and/or composition studies;
- GM adventitious presence issues 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 first report for the 2006/2007 year and covers the period from July to December 2006.

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:***

CODEX: Codex Alimentarius Commission

EU: European Union

FAO: Food and Agricultural Organization of the United Nations

FSANZ: Food Standards Australia New Zealand

EFSA: European Food Safety Authority

UKFSA: United Kingdom Food Standards Agency

USDA: United States Department of Agriculture

USFDA: United States Food and Drug Administration

WHO: World Health Organization

## 2 PART A: FOODS FROM GENETICALLY MODIFIED ORGANISMS

### 2.1 NOVEL TECHNIQUES FOR DEVELOPING GM PLANTS

#### 2.1.1 *Cre/lox technologies for plant transformation*

The introduction of foreign genes into plants is one of the approaches now being taken to engineer new traits and overcome the barriers to classical plant breeding that include sexual compatibility. Genetic engineering makes sexual and physiological barriers irrelevant and the rapidly expanding number of genetically modified crops being grown world-wide is testament to the power of this methodology. Two ongoing issues with the genetic engineering of crops are:

- i. Integration of transgenes into plants predominantly via ‘illegitimate recombination’. Whilst a number of methodologies are utilised to introduce foreign genes into plants, e.g. *Agrobacterium*-mediated transformation and biolistic particle bombardment, these methods result in random localisation of the introduced gene construct in the plant genome. This can result in the generation of complex integration structures which may be unreliably expressed in the engineered plant and are often too complex to easily characterise. Stable transgene expression requires precise, single-copy integration of the gene construct.
- ii. The persistence within the transgenic crop of extraneous portions of DNA not necessary for the ongoing expression of the transgene within the engineered plant. For example, vector backbone sequences and selectable marker genes. Many transformation methodologies rely on the use of selectable marker systems to identify engineered plants in the early stages of development. Often the choice of selectable marker has been for resistance to antibiotics. The presence of antibiotic resistance genes in genetically engineered crops has been an increasing public cause of concern, both from an environmental and a public health perspective. Whilst there is little scientific evidence to support a risk from extraneous DNA sequences in the food chain, the European Commission has gone so far as to recommend the minimisation of such sequences in transgenic crops.

*“Joint SCF/SCP/SCAN GM/NF WG GM-NF WG/Guide/005  
Opinions of the Scientific Steering Committee  
Preliminary Guidance document on the information needed for the risk  
assessment of genetically modified plants and derived food and feed (2002)*

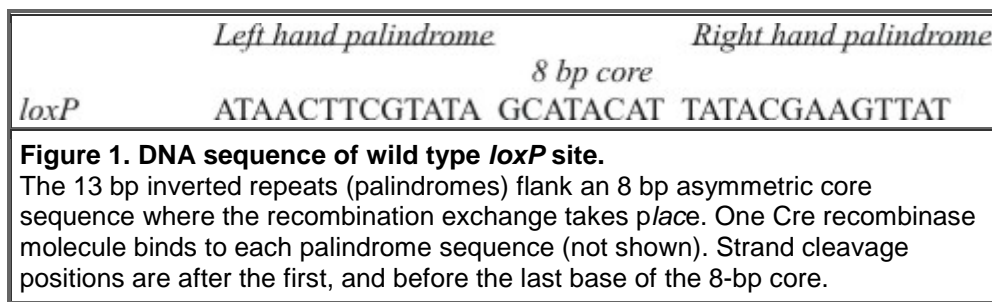
*1.5 Risk assessment related to the genes inserted  
Notifiers are encouraged to develop “clean vector” technologies ... in which  
genes extraneous to the successful deployment of the target transformation  
event are removed. Whenever possible, notifiers are encouraged to develop,  
for commercial release, those transgenic lines in which only DNA essential to  
the modification of the trait in question is transferred to the plant.”*

[http://ec.europa.eu/food/fs/sc/ssc/guidance\\_gmo\\_en.pdf](http://ec.europa.eu/food/fs/sc/ssc/guidance_gmo_en.pdf)

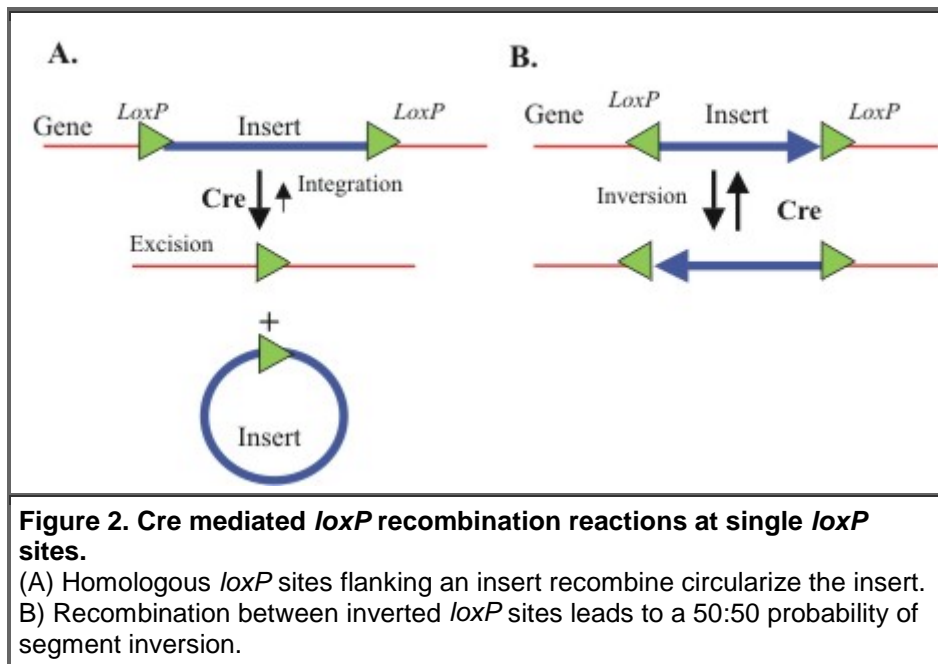
The Cre/*lox* system for precise integration and removal of DNA sequences is becoming increasingly utilised in plant biotechnology to overcome these issues. During the reporting period an extensive review on the use of Cre/*lox* technologies for plant transformation was published (Srivastava and Nicholson, 2006).

### Background to Cre/*lox* system

The Cre/*lox* recombination system is derived from the bacteriophage P1. This bacteriophage is maintained inside *E.coli* bacterial cells as a single copy, circular plasmid DNA molecule. The Cre/*lox* system allows the bacteriophage to separate copies of the DNA molecule during replication. The *cre* gene (cyclization recombination) encodes a site-specific DNA recombinase enzyme. This enzyme, Cre, can recombine DNA when it locates specific sites in a DNA molecule. The target for the Cre enzyme is *loxP* (locus cross-over P1). When cells that have *loxP* sites in their genome also express the Cre protein a reciprocal recombination occurs between the *loxP* sites. *LoxP* sites are made up of 34 base pairs of DNA, containing two 13 base pair inverted repeats and an 8 base pair core sequence (see Fig 1, taken from Norman and MacInnes)

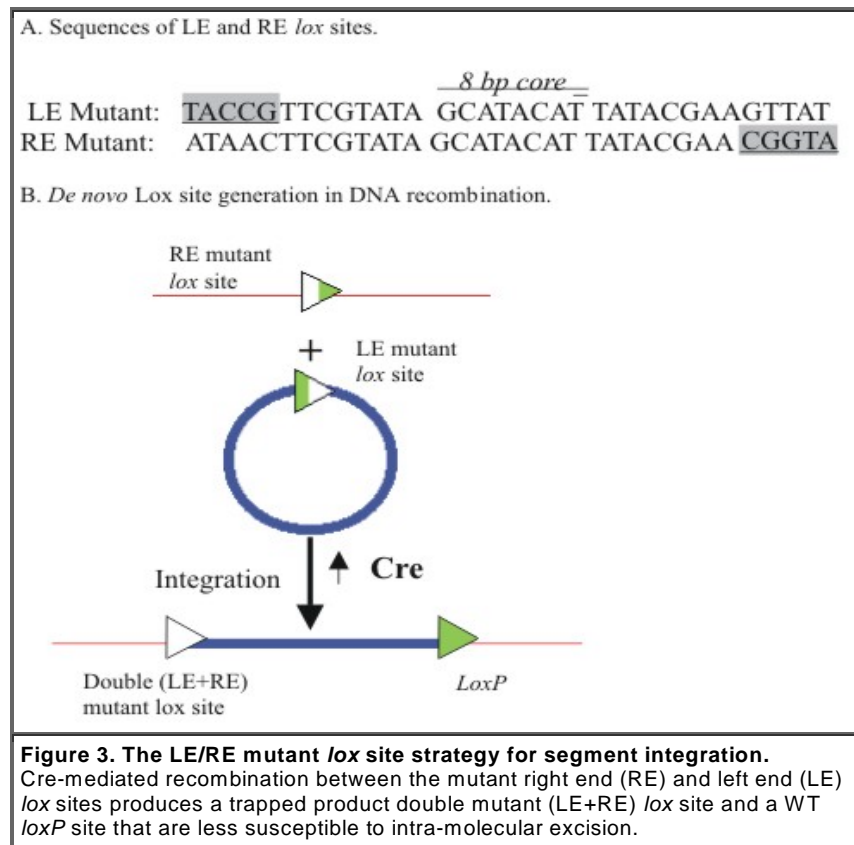


The asymmetry of the 8 base pair core sequence confers directionality on the *loxP* site. This has implications for the functioning of adjacent sites in recombination. The Cre enzyme is able to catalyse intra-molecular integration and excision events as well as inter-molecular DNA exchange. When two adjacent *loxP* sites are in the same orientation the Cre enzyme will preferentially produce an excision reaction with circularisation of the DNA between the sites. When two *loxP* sites are in reverse orientation to each other an intra-molecular inversion of the insert can occur with a 50:50 probability (see Fig 2, taken from Norman and MacInnes).



When a Cre-mediated excision event has taken place a single *loxP* site is left behind as a 'footprint' in the host DNA.

While Cre recombinase can also catalyse the insertion of DNA into a *loxP* site (i.e: an integration reaction) this reaction is inefficient as the inserted DNA is then flanked by two *loxP* sites and is immediately excised again. So, while the integration reaction is reversible, the excision reaction is practically irreversible. Studies on mutant *loxP* sites, some of which are naturally occurring, has provided information on the ability of homologous and heterologous *loxP* sequences to recombine. Use has been made of such mutations in *loxP* to enable integration of DNA at a target site without immediate re-excision (see Fig 3, Norman and MacInnes).



2.1.1.1 ‘*Cre/lox technologies for plant transformation*’, Srivastava and Nicholson, 2006.

This review considers the use of the *Cre/lox* recombination system to remove marker genes from transgenic plants as well as to enable site-specific integration of transgene sequences.

*Removal of marker genes*

In many plant systems it is not logistically feasible to try to engineer traits into the plant without the use of some selectable marker system. The low frequency of transgene integration, the need to grow large numbers of plants to a stage where the introduced transgene can be selected for and the logistics of such large-scale experiments precludes marker-less production of many transgenic crops. *Cre/lox* recombination can be used to remove a marker gene from a transgenic plant after the initial stage of transformation, and once transgenic lines have been identified. Marker genes, such as an antibiotic resistance gene, are flanked by *loxP* sites within the integration cassette. If the *cre* gene is expressed within the engineered system the sequences between the *loxP* sites are then excised. The excised, circular DNA molecule is unable to replicate and is believed to be lost in subsequent cell divisions. Whilst recent studies on *Cre/lox* excision of markers genes in the wheat genome are presented that indicate that the excised circle can be maintained as an extra-chromosomal element, the mechanism for this is not known and the rarity of the event is concluded to limit its impact on the effectiveness of the *Cre/lox* system.

Along with the introduction of a marker gene flanked by *loxP* site, a system needs to be engineered in which Cre recombinase activity is provided to enable the excision event. Several systems are described in the review:

- i. A transgenic line containing a marker gene flanked by *loxP* sites can be re-transformed with the *cre* gene. This approach is relatively time-consuming but has been shown in tobacco to generate marker-free transgenic lines with high efficiency.
- ii. A Cre expressing transgenic line can be generated and used for traditional crossing with a transgenic line containing a *loxP*-flanked marker gene construct. The *Cre/lox* system should be complete in the F1 progeny of crossing, resulting in excision of the *loxP* flanked marker gene. The *cre* locus could then be removed by segregation in subsequent generations. While a simpler approach than the double transformation system outlined above, the efficiency of the crossing method has been shown to be highly variable.

The functionality of the *Cre/lox* system has been demonstrated in a number of plant systems including several important cereal crops; rice, wheat and maize.

An important extension of the *Cre/lox* system for the removal of marker genes in transgenic plants is the use of inducible promoter systems. The review describes development of these systems. Inducible systems are considered to be particularly useful for vegetatively propagated crops, where crossing systems are inappropriate. Inducible promoter systems are described as auto-excision systems. Typically, both a marker gene and the *cre* gene are flanked by *loxP* sites. The *cre* gene is under the control of an inducible promoter and when activated induces the excision of the entire region between the *loxP* sites. Inducible promoters described in the review that are able to function successfully in auto-excision *Cre/lox* systems in plants include: (i) chemical-induced promoters (e.g: estrogen-inducible promoter elements) in *Arabidopsis* and rice, and (ii) heat-inducible promoters in *Arabidopsis*, tobacco, rice and maize.

The *Cre/lox* system has also been used to successfully eliminate marker genes from plastid genomes in tobacco. There are approximately 10,000 copies of plastid genome per leaf cell in tobacco. A nuclear expressed *cre* gene product has been used to rapidly eliminate *loxP*-flanked marker genes from all plastid genomes in a transplastomic tobacco line. The nuclear *cre* gene could then be removed from the plant line by traditional crossing methods. Transplastomic generation of GM plants has been proposed as a method to limit unintentional environmental transfer of transgenes as plastid genomes are maternally inherited in most plant species and so are not found in the pollen of a transgenic line.

#### *Site-specific gene integration using Cre/lox*

Controlling transgene integration structure and site has several advantages in the generation of transgenic plants. A precise, single copy integration of the transgene construct is likely to be stable through successive generations. It is also advantageous to target integration into favourable genomic locations for predictable genomic expression. For example, localisation of a transgene into a transcriptionally active region of a genome, rather than into a 'silent' region should help facilitate expression of the transgene. The *Cre/lox* recombination system can be utilised to target transgene integration. The review outlines the basic strategy for targeted integration and describes instances of its successful use.

The basic strategy for a targeted integration is to firstly develop a target, or host, line containing a *loxP* target construct. The target construct contains a portion of a gene construct, for example the coding region of a marker gene without a promoter element, and a single *loxP* site. This line is generated using traditional transformation strategies that result in random, and often multi-copy, integration events and a single copy integration event must be identified from within a large pool of transgenic lines. Once the target line has been identified it is retransformed with a *lox*-integration construct. The integration construct contains the remainder of the gene construct, for example the promoter element, along with a second *loxP* site. Cre enzyme activity can be provided in one of two ways:

- i. The *cre* gene can be incorporated in the host-line target construct
- ii. The *cre* gene can be expressed from a co-introduced plasmid

In the presence of Cre enzyme a site-specific recombination occurs between the *loxP* sites on the target construct within the host line and the *loxP* site on the integration construct. A complete integration structure, for example marker gene plus promoter element, is generated. The use of mutant *loxP* sites has been important in developing this site-specific integration system as it overcomes the potential issue of re-excision of the integrated construct.

Cre-mediated site-specific integration has been demonstrated in a range of plant systems, including *Arabidopsis*, rice and maize. The system has been used successfully with both *Agrobacterium*-mediated T-DNA delivery and with direct DNA delivery (e.g. biolistic particle bombardment and PEG-mediated protoplast transformation). Interestingly the efficiency of site-specific integration has been shown to be higher with direct DNA delivery methods. It is suggested that T-DNA processing in plants exposed to *Agrobacterium*-mediated transformation may interfere with the Cre/*lox* reaction, resulting in lower efficiency of integration. It is pointed out in the review that while site-specific integration is a selectable event, non-targeted, random integrations may also occur in the background. Two types of integrant line may therefore result, possessing either (i) site-specific integration without random integrations, or (ii) site-specific integration with random integrations. Molecular characterisation of the integrant lines, for example using Southern hybridisations, is required to distinguish between these two types of line.

Studies are described that analyse the expression patterns of genes introduced into plants by Cre-mediated site-specific integration. Not unexpectedly expression levels vary for the same integration constructs inserted into different loci. However, variation has also been seen in expression of an integration construct in seedlings from a line with a single integration site. DNA methylation, resulting in gene silencing, has been concluded to be responsible for this observation. Therefore, whilst site-specific integration can be used to generate stable expression in transgenic lines, it is necessary to perform gene expression analysis to eliminate unstable lines from a transformant pool. Gene expression from site-specific integration loci has been shown to be stable through successive generations and also to correlate directly with allelic gene dosage, i.e. expression levels double in the homozygous state.

The conversion of a complex integration locus into a single-copy locus is also made possible using Cre-mediated recombination. For some plant species recalcitrant to transformation, e.g. wheat, soybean and maize, it is difficult to generate the necessary number of transgenics to obtain single-locus integration. Similarly, biolistic transformation is noted for the number of rearranged and duplicated insertion events that occur. An integration cassette can be designed that has multiple *loxP* sites. Under the influence of Cre recombinase complex

integration events, where more than one copy of the cassette have been introduced, can be resolved to a single copy locus by utilising the excision characteristics of the *loxP* site orientations.

#### *Issues to consider when using Cre/lox technology*

Cre ‘toxicity’ has been observed by some researchers studying Cre/lox technology in plant systems. Stunted phenotypes and growth inhibition, along with chromosomal rearrangements have been seen in several different plant species expressing Cre recombinase. Cre toxicity has been shown to correlate with the levels of Cre protein in the plants, with Cre toxicity decreasing with decreasing levels of Cre enzyme activity. It is suggested that the use of strong constitutive promoters to drive *cre* gene expression is more likely to result in Cre phenotypes than the use of native plant or inducible promoters. A possible cause of the aberrant phenotypes occasionally seen in transgenic lines expressing Cre at high levels is the presence of *lox*-like or pseudo-*lox* sites in the plant genome that may interact with the Cre recombinase to cause intrachromosomal inversions or excisions. Such sites have been found in the human and mouse genomes and have been shown to support Cre-mediated recombination *in vitro*, albeit at very low frequency. Given that most crop plants have large genomes, the potential for pseudo-*lox* sites to be present needs to be considered when utilizing Cre/lox systems for generation of transgenic crop plants. Suggestions to minimise the potential for Cre toxicity to occur in plants are (i) to limit Cre levels by the use of low level, endogenous and inducible promoters, and (ii) to use excision and crossing strategies to remove the *cre* gene from the transgenic line as soon as it is no longer required for integration or excision events. This latter strategy should also help to alleviate any public health concern about the presence of Cre recombinase in crop plants used as food.

The ‘footprint’ *loxP* site left behind in the plant genome after an excision event has been suggested to be a potential risk associated with this technology. Public submissions addressed in the recently released FSANZ Final Assessment Report for Food derived from High Lysine corn line LY038 include concern over the presence of a *loxP* site in the plant genome after excision of the selectable marker gene for kanamycin resistance. The review suggests that the presence of pseudo-*lox* sites in plant genomes could be considered as a useful tool for integration strategies rather than as a potential hazard. It may be possible to utilise such sites to direct integration events rather than having to first introduce a *loxP* site.

The use of endogenous pseudo-*lox* sites could be coupled to the recent development of ‘endogenous’ transfer constructs for the generation of engineered traits in plants (see report FW0518, July – December 2004, Section 2.8.2). Generation of plant lines using these technologies could test the definition of ‘transgenic’ and ‘genetically modified’. They would be difficult to test for in the food chain due to the absence of ‘foreign DNA’ markers.

In summary the use of Cre/lox technologies in plant transformation is well covered in this review, including a comprehensive description of the development of the technology. Examples of uses are described and potential uses expanded on. To date marker-deletion by Cre-mediated excision has been used in some commercial transgenic lines, for example High Lysine corn line LY038. As the site-specific gene integration strategy doesn’t allow for removal of marker genes and extraneous DNA sequences it is not yet suitable for commercial application. However, strategies are being developed to enable the generation of a ‘clean’ integration loci using Cre-mediated recombination. While patent and intellectual property

concerns have prompted the search for alternative recombination systems, the efficiency and precision of the Cre/lox system is to date unmatched and the system is likely to be increasingly utilised in the generation of commercial transgenic plants.

Sources:

Norman, A and MacInnes, H. Genetic engineering of embryonic stem cells via site-directed DNA recombination. *Online Reviews in Undergraduate Research*, Issue 1.  
[http://www.ruf.rice.edu/~rur/issue1\\_files/norman.html](http://www.ruf.rice.edu/~rur/issue1_files/norman.html)

Srivastava, V and Nicholson, S.J. (2006). Cre/lox technologies for plant transformation. *CAB Reviews: Perspectives in Agriculture, Veterinary Science, Nutrition and Natural Resources* 1(034): 1-12.

## 2.2 GM FOOD SAFETY

### 2.2.1 *The use of –omic techniques for GM food safety analysis*

Various regulatory controls for the release of GM products into the food market have been in place for a number of years. In New Zealand and Australia these regulations are under the direction of FSANZ, and in the European Union by the European Food Safety Authority (EFSA). Regulations under both these bodies require that a thorough and extensive safety assessment be undertaken before a new GM crop can be grown or used as food. Details of the current requirements for these assessments can be seen at:

EFSA - [www.efsa.eu.int/660/guidance\\_docfinal1.pdf](http://www.efsa.eu.int/660/guidance_docfinal1.pdf), and  
FSANZ - <http://www.foodstandards.gov.au/srcfiles/Application%20Format%20-%20GM%20June%202005.doc>.

These regulations are underpinned by the Codex ‘Guidelines for the Conduct of Food Safety Assessments of Foods Derived from Recombinant –DNA Plants’  
([ftp://ftp.fao.org/es/esn/food/guide\\_plants\\_en.pdf](ftp://ftp.fao.org/es/esn/food/guide_plants_en.pdf)).

In all cases the risk analysis is based on the concept of ‘substantial equivalence’. Substantial equivalence relies on the rationale that an existing organism, used as food or feed, that has a history of safe use, can be used as a comparator when assessing the safety of GM food/feed. This comparator is usually either the parent plant line used in the genetic modification or a non-transgenic (null) ‘segregant’ taken from the transformation experiment or from post-transformation breeding experiments. Presently the assessments are targeted at specific factors such as the nature of the genetic modifications, molecular characterisation of these modifications in the GM plant, the potential for introduced protein(s) in the plant to have toxic or allergenic activity, changes in the nutritional composition of the plant as a food, and any unintended changes in the plant. Unintended effects are any differences observed between the GM plant and its comparator that go beyond those expected from the introduction of the target transgene. Currently these unintended changes are assessed in a somewhat limited manner by assessment for targeted factors (eg: specific nutrient and antinutrient composition) and by bio-informatic assessment of the novel protein in the transgenic plant. Recently the EFSA has recommended that further exploration of profiling approaches be undertaken. Profiling methods are not intended to replace existing assessment

analyses but to confirm and supplement this data. This has also been recognised by FAO/WHO in 2000 and the UK GM Science Review panel in 2003. Profiling techniques include the -omic approaches of transcriptomics, proteomics and metabolomics. Used separately or in combination these techniques could enhance the current safety assessment of GM foods.

In 2001, in response to these recommendations, the UKFSA commissioned a major research programme to study the use of molecular profiling techniques in the safety assessment of GM food crops. A summary of the studies was released in 2006.

#### 2.2.1.1 'Use of -omic techniques for GM food safety assessment'. Molnar, (2006).

In this report experiments using three types of -omic techniques (transcriptomics, proteomics and metabolomics) are summarised. These techniques all rely on the large scale profiling of molecules from a target plant and an appropriate comparator. The 'Central Dogma Theory of Genetics' states that DNA is transcribed into RNA that is then translated into protein. Proteins are intrinsic in the establishment and maintenance of biochemical pathways in an organism that lead to the production/turnover of various metabolites. Whilst utilising different methodologies the -omic technologies are inter-related in that:

- Transcriptomics looks at changes in the transcriptome (the entire complement of RNA produced by DNA transcription) of a cell, tissue or organism at a particular time point.
- Proteomics studies the total protein complement of a cell/tissue/organism (the proteome).
- Metabolomics is the analysis of the entire complement of small (low molecular weight, <1500 daltons) molecules in a cell/tissue/organism.

The report summarises research undertaken at several research institutions to assess whether these methodologies are suitable for refining the current safety assessment of GM crops.

#### Transcriptomics

Two different methodologies to determine if the expression of genes differs in GM plants compared to non-GM counterparts are described:

- i. Microarray technology: This method utilises a commercial or in-house array 'chip', on which is spotted a large number of sequences representing individual gene transcripts. Depending on the plant species being studied, these chips may be whole-genome representative, or represent only a sub-set of expressed gene sequences. Examples of 'chips' are a commercially available one for barley (which can also be used for wheat), while Dutch researchers have generated their own array for potato, as commercial chips were unavailable. Not all commercial arrays contain whole-genome representation, depending on the status of genome sequencing projects for the particular plant species. Gene expression 'chips' are then used to identify which genes are being transcribed in a target plant tissue at a particular time point and comparison can be made between a GM plant and an appropriate non-GM comparator.
- ii. SAGE (Serial Analysis of Gene Expression): This method captures all of the transcribed RNAs from a sample, "rewrites" them into DNA, and cuts a small, unique fourteen-base tag from each one. A number of tags are joined together into one long molecule, which is then sequenced. Sequences can then be compared to known gene

sequences for the organism. Even if a sequence function is unknown it can still be compared to the same sequence from a comparator plant and any changes in levels of gene expression can be determined. SAGE is an 'open' system that makes no assumptions about the sequences that will be tagged and requires no prior knowledge of gene sequence.

The report summarises studies at the Scottish Crop Research Institute, which investigated the use of both microarray and SAGE techniques for GM food risk assessment. Gene expression patterns in transgenic barley were compared to non-GM lines and null-segregant lines. Both methods were successful at detecting changes in the transcriptome of the plants but it was concluded that it was difficult to directly compare the two methods. SAGE identified 58 genes showing a 5-fold difference in abundance between GM and null-segregant lines. With a different GM line, microarray detected approximately 8% of genes showing a difference in levels of expression. One of the limitations of both of these methods is concluded to be the 'fluidity' of the transcriptome itself. Not all transcribed sequences are necessarily translated into proteins. Thus the analysis of changes to the transcriptome does not necessarily reflect a change in the profile of 'end-products'.

### Proteomics

Various techniques were tested to determine if analysis of the proteome of GM plants could confidently be used to determine differences in protein composition to a non-GM counterpart.

- i. Researchers at Rothamstead Research used a 2-dimensional gel electrophoretic separation technique to analyse the difference in protein composition between a GM wheat line and a non-GM comparator. One of the main targets of the study was to produce images of 2-D protein separation that could be compared computationally. It was determined that establishment of and care in standard operational procedures (SOPs) for extraction of proteins from flour samples and in the running of the 2-D gels was critical for the generation of protein 'fingerprints' that could be compared using digital image analysis software. Differences in protein profiles between GM and non-GM lines were seen, however, the greatest effect was from natural environmental variation and was not specific for the GM line.
- ii. Multidimensional Protein Identification Technology (MudPIT) was assessed at the Institute of Food Research. The model plant *Arabidopsis* was modified by the insertion of a bacterial colourimetric marker gene – beta glucuronidase (*gus*). MudPIT has an increased sensitivity and higher sample throughput than 2D-gels. Peptides isolated from the test plants are separated by 2-D liquid chromatography and then injected directly into a mass spectrometer. The researchers were able to easily identify the GUS protein using this method. It was concluded that further development of the method could see it utilised for safety assessment of GM foods.

## Metabolomics

Unlike the targeted analyses currently used in GM food safety assessment, metabolomics has the ability to analyse hundreds of molecules at the same time. Two main techniques were assessed by researchers and summarised in the report:

- i. Nuclear Magnetic Resonance (NMR): Studies from two research groups who investigated the use of NMR to profile the metabolites in GM plants are presented in the review. Researchers at the Central Science Laboratory (CSL) considered NMR a rapid, reproducible and robust technique to generate an overall metabolite profile from a crop. They found the method useful for comparing GM to non-GM wheat and barley. NMR results indicated one unidentified, possibly novel metabolite was increased in all 5 transgenic barley lines studied. Overall there were fewer changes seen in the metabolome of GM wheat than of GM barley. It is suggested that this may a consequence of barley having a diploid genome, whereas wheat is hexaploid. The more genome copies present in a plant the more likely changes from one modified genome are to be out-competed by the remaining genomes. It is likely that this level of difference would not have been detected using current safety assessment methods. However, differences were also detected when comparing both transgenic lines and null-segregants of these lines (plants subject to the same genetic engineering process, but not containing a transgene), with wild type lines. This would suggest that at least some differences seen in the metabolome of GM and null-segregants may relate to variation induced during the tissue culture phase of experimental procedures and not as a result of the introduction of the transgene *per se*. Such somaclonal variation is well known as a potential consequence of *in vitro* manipulations of plant material.
- ii. Mass spectrometry (MS): MS techniques were used by several groups of researchers to analyse the metabolome of transgenic potato. Using a number of different MS-based instruments at more than one site researchers at the University of Wales and of Golm (Germany) obtained results that were comparable. They determined that the particular transgenic line had an identical metabolome to the non-GM parent line with the exception of the engineered trait – an increase in levels of the storage carbohydrate fructan. Similar results were obtained at the Scottish Research Institute with the addition of information on the importance of sample preparation to metabolomic analysis. These researchers showed that differences in the metabolome profile could be detected even throughout the same potato tuber. Overall the method of gas chromatography-MS (GC-MS) was considered to be the best method for routine generation of metabolite profiles from plants. It was determined to be a faster and more reproducible method compared to the alternative use of liquid chromatograph-MS (LC-MS).

The review concludes that methods developed in this extensive research programme were successful at detecting unintended changes resulting from transgene insertion into plants at the levels of RNA, proteins and metabolites. However, many of these changes could be due to somaclonal variation resulting from the *in vitro* manipulation of plants rather than the presence of an inserted transgene. Differences between plants grown in different environments and even different cultivars of the same species (e.g. potato) grown in the same environment were often greater than the effect of the transgene itself. Whilst based on analysis of a greater number of targets, these results are in agreement with those of many standard safety assessments for GM foods. Variations determined in compositional studies

are most often within the range of natural, environmental and/or cultivar variations and not linked to the presence of a transgene.

Research in this programme also highlighted the importance of quality control for these types of large-scale determinant assays. Care needs to be taken in the choice of and collection of sample material and in the standardisation of experimental SOPs so as not to introduce artificial variation into the system. Data generated by these types of large-scale assays is huge and needs appropriate data handling and expert statistical analysis.

Recent safety assessments for GM foods reported on by FSANZ as a consequence of application for approval to market specific GM crops have received public submissions urging the incorporation of -omics techniques as mandatory additional assays required before any approval is given. The results of the extensive set of experiments summarised in the above review suggest that while these techniques have the potential to augment safety assessments there is still a lot of establishment and developmental research that needs to be done before these assays can become routine. Much of this work centres round the establishment of databases of data to inform results. The large potential for 'natural' variation in the profiles of molecules obtained from plants, particularly for metabolites, means that extensive data needs to be collected to determine levels of natural variation, and what factors, such as environmental exposures, may influence them. This information is unlikely to be generic, as variation profiles are likely to be plant, species, cultivar and even tissue specific. Compositional assays used in current safety assessments are generally for analytes that have known level ranges in plants and often have documented toxicity ranges. The large numbers of targets analysed by -omics do not generally have known natural 'variation' ranges and are themselves often unidentified compounds. Extensive background data needs to be generated for a range of plants before reliable results on unintended effects can be obtained for GM plants using these methodologies. Once this information is collated and becomes accessible in public domain databases the use of -omics technologies may well prove to be a useful addition to the current assays that are required for safety assessments for GM foods.

Source: Molnar, S. (2006). Use of -omics technologies for GM food safety assessment. *Food Science and Technology* 19(4): 20-22.

### ***2.2.2 Evaluation of human allergenic potential of novel proteins in GM foods***

The commercialisation of GM crops to date has concentrated on a selected range of crops and introduced traits. Corn and soybean products make up the majority of approved GM foods and contain GM traits for insect and/or herbicide resistance. Rice, bi-products from cotton (oil and linters) and some smaller crops e.g. papaya, are also present in the global market. These crops are often not perceived as having any direct consumer benefit, with the agronomic benefits being obtained by the farmer. However, there is continuing research and development world-wide to generate a wider range of crops with many more engineered traits that may be of direct benefit to the consumer. Such traits include enhanced nutritional and nutraceutical composition, improved storage characteristics, resistance to spoilage, improved flavour and appearance and the elimination of naturally occurring toxins, including allergens. The safety evaluation of this increasingly diverse range of GM foods is going to be critically important in ensuring public health and the concomitant public acceptance of these crops.

Development of genetically engineered food largely results in the production of novel protein in a crop. To date identified food components that cause allergenic reactions in humans are virtually all proteins, so the safety assessment of novel proteins in GM foods is a key component to the overall safety assessment process.

#### Background to food allergies

Food allergies affect an estimated 3-4% of the population and while they can affect all age groups, infants and young children often show a higher prevalence than adults. In general eight food groups are responsible for more than 90% of all food allergies world-wide. These are milk, eggs, fish, crustacean shellfish, peanuts, tree nuts (eg; almond and brazil nuts), soybeans and wheat. Some other specific food allergies can occur in some geographical areas, eg: celery allergy in Europe, probably as a result of cross-reactive pollen allergies in these areas coupled with specific cultural dietary practices. Genetic differences between individuals have been suggested to contribute to individual susceptibility to food allergies, however, no evidence yet exists to substantiate suggestions of any racial or ethnic differences beyond those of cultural dietary exposure and practices.

Food allergy is a complex immune response to an otherwise innocuous dietary protein. Different proteins have different structures and in an allergic reaction immunoglobulin (IgE) antibodies bind specifically and with high affinity to structures (epitopes) on the allergenic protein. IgE is usually bound, via high-affinity receptors, to mast cells. Mast cells are present in most tissues in the vicinity of blood vessels, and are especially prominent near the boundaries between the outside world and the internal system, such as the skin, mucosa of the lungs and digestive tract as well as in the mouth and nose. Allergens that are able to bind specifically to two or more IgE molecules will cross-link the cell receptors to which these IgE molecules are bound, inducing intracellular signals that result in the release of histamine, and sometime leucotriens. Release of these molecules in turn results in an inflammatory response. This can vary from delayed, or chronic responses, which also involve a T-cell response, through to acute local and systemic symptoms. Anaphylaxis and possible death can occur in extreme acute responses. Diagnosis of the causal agent of an allergic response can be difficult as people eat a wide range of foods, and the level of response can be affected by responses to contact or inhaled allergens as well. Once a food allergenic response has been

identified in an individual, avoidance of the source is the only way most people can remain symptom free.

The potential for introduction into GM foods of novel proteins that have not traditionally been a part of our food chain increases the need to have reliable assessments available for allergenicity of novel proteins. Unfortunately no single test currently exists to fully predict the potential allergenicity of any specific novel protein. An array of tests has therefore been developed for this purpose. During the reporting period a review of the development of methodologies for evaluating the human allergenic potential of novel proteins was published by S. Taylor, University of Nebraska, Food Allergy Research and Resource Programme. Information provided in this review is summarised in this report and ongoing issues are discussed.

#### *2.2.2.1 Review of the development of methodology for evaluating the human allergenic potential of novel proteins, S. Taylor, 2006.*

Assessment of the allergenicity of the novel proteins in GM foods was recognised as an important issue by the USDA and other regulatory agencies in the early 1990s. The development of an engineered soybean variety for improved nutritional qualities for use as animal feed was central to this issue. The soybean was engineered for enhanced methionine content by introduction of a methionine-rich protein from Brazil nuts. Whilst at the time Brazil nuts were known to cause food allergies in some individuals the protein responsible had not been identified. The developers of the engineered soybean line recognised that they needed to determine if the Brazil nut protein in their line was allergenic and, using sera from individuals with known Brazil nut allergy, determined that this high-methionine protein is the likely major allergen of Brazil nuts. This is still the only example of an engineered crop known to have allergenic potential and is an example of an assessment strategy that was able to identify an allergenicity risk from a novel protein in a GM crop. On the basis of the assessment the producer decided not to commercialise this soybean line.

Attempts were then made to establish effective approaches for allergenicity assessment of genetically engineered crops. In 1996 the International Life Sciences Institute and the International Food Biotechnology Council (ILSI/IFBC) proposed a decision-making tree approach that utilised several different tests. The rationale was that overall predictability of allergenicity would be improved by a combination of tests. This approach provided reasonable assurance that a novel protein was unlikely to be allergenic, however, it was subject to some peer criticism. In response to this criticism, in 2001, the FAO and WHO assembled an expert panel to consider an alternative approach. The resulting 2001 FAO/WHO decision tree (see Annex A) contained many of the same elements as the ILSI/IFBC tree, but incorporated some additional approaches. Criticism was also levelled at this new approach and was directed particularly at the inclusion of target serum screening tests and use of animal model systems. The Codex Ad Hoc Intergovernmental Task Force on Foods Derived from Biotechnology concluded that these two methods were not well enough developed to be used with confidence by governmental regulatory agencies.

Currently allergenicity assessment of GM foods is based on elements of the FAO/WHO tree and incorporates an evaluation of the source of the introduced gene, amino acid sequence homology to known allergens, specific serum screening and comparative pepsin resistance.

A weight-of-evidence approach is now supported over a defined decision-tree approach. The rationale and details of these assessments are summarised below:

i. Source of the novel gene

If the novel gene in a GM food is from a source that is known to cause food allergies then the decision process presumes that the gene may be allergenic unless proven otherwise by the other testing methodologies. Sources that would cause concern would include genes from the following plants: peanuts, soybeans, tree nuts, and wheat, and from the animal sources: eggs, milk, fish and crustacean. It is important to also consider whether the source is known to be responsible for environmental allergies as some environmental allergens, e.g. pollen, are cross-reactive with food allergens. To date most of the genes used to generate transgenic crops have been obtained from sources with no history of allergenicity, however this does not completely preclude an allergenic potential.

ii. Sequence homology to known allergens (bioinformatics)

A number of public domain databases are available that contain the amino acid sequence of a range of known environmental and food allergens (e.g. [www.allergenonline.com](http://www.allergenonline.com)). Thus the amino acid sequence of the novel protein introduced into a GM food can be compared to known sequences of food and environmental allergens. If a sufficient degree of homology exists then the possibility of the novel protein inducing a response in individuals with an allergy to the known allergen must be considered. The criteria used to determine a significant homology have been subject to debate. The ILSI/IFBC system suggests a match of at least 8 contiguous identical amino acids. FAO/WHO proposed a match of at least 6 contiguous identical amino acids, or sequence identity of at least 35% over a sliding-window of 80 amino acids. The 6 identical amino acids system has been demonstrated in wheat to give numerous false positive matches. The guidelines of the Codex Ad Hoc Intergovernmental Task Force on Foods Derived from Biotechnology therefore suggest that a 35% homology over an 80 amino acid sliding window be used as a measure of significant homology and that exact matches over 6-8 amino acids may be useful indicators of allergenic potential, but must be validated by other methods. The 35% identity over 80 amino acids is intended to identify proteins that share a structural homology with a known allergen. It has been shown that many plant allergens fall into common functional groups and that those with the most similar structures are often cross-reactive in inducing allergic responses. There are several limitations to the use of bioinformatics in the assessment of the potential for novel proteins in GM food to be allergenic. Existing knowledge of the identity and sequences of known environmental and food allergens is one limitation. However, databases are continually growing in size and at least 1100 allergens are now characterised to varying levels. Another limitation is that a high degree of amino acid sequence homology can occur without clinically relevant cross-reactivity. For example, chicken and shrimp tropomyosins share about 60% homology but there is no IgE cross-reactivity between these foods. This limitation is likely to result in false positive rather than false negative assumptions about the potential allergenicity of novel proteins in GM foods, which would keep non-allergenic GM foods out of the market rather than resulting in the release of ones with potential for allergenicity.

iii. Specific serum screening

If the results of the analyses in (i) and/or (ii) above are suggestive of a potential for allergenicity of a novel protein in a GM food, then a specific serum screening test is advised. Specific serum screening involves testing whether the novel protein is immunoreactive with the sera of individuals allergic to the source material. If the transgene is derived from a known allergenic source, specific serum testing is particularly important. Also, not all structures of allergens from known allergenic sources are characterised and as such information may not be available for bioinformatics validation of allergenicity of the novel protein. The specific serum test is dependant on the availability of sera from well characterised patients and this can be a limitation for use of this methodology. False positive results are also a concern with specific serum testing. For example, many plant proteins have carbohydrate groups attached to them and IgE has been documented to bind to carbohydrates. The clinical significance of such binding in eliciting an allergic cross-reactivity response is not clear, but the possible binding of IgE to carbohydrate moieties needs to be eliminated from any positive specific serum tests. To overcome the potential for false positive results it has been suggested that further clinical tests, including skin pricks and double-blind, placebo-controlled oral challenges be used to confirm the allergenicity of a novel protein in a GM food. However, in many countries the ethics of such human trial tests are likely to be problematic to overcome.

iv. Resistance to pepsin

As proteins from food pass through the digestive system they are subject to hydrolytic break down by digestive enzymes. In general, known food allergen proteins show a greater stability to enzymic hydrolysis in simulated gastric and digestive model systems than known non-allergenic proteins. Therefore allergenic proteins are likely to reach the intestinal tract in a form that is sufficiently intact to allow them to bind to IgEs and elicit an allergic response. Digestive stability in simulated *in vitro* systems was proposed as a criterion for the assessment of allergenic potential of proteins introduced into GM foods. However, it is well known that humans vary widely in their digestive capacity and current *in vitro* models do not encompass the entire range of human digestive capacity. Instead, the comparative resistance of a protein to breakdown by the enzyme pepsin (a protein degrading enzyme found in the stomach) has been accepted as an assessment tool. Novel proteins resistant to pepsin are considered to be more likely to have allergenic potential than those that are rapidly broken down by pepsin. However, the conditions used in the pepsin resistance assay have been shown to be critical and a SOP for this assay is important for its use in allergenicity assessment. Some allergenic proteins have also been shown to be sensitive to pepsin and so would pass the pepsin resistance test. These particular allergens tend to be ones that are cross-reactive with known pollen allergens and so would be likely to be detected during bioinformatics assessment.

The limitations of the above four assessment systems highlight the need to determine the allergenic potential of a novel protein in a GM food by the weight-of-evidence of a number of tests and not from the results of one test alone. The weight-of-evidence approach provides a reasonable assurance of allergenic potential of a novel protein and should be strengthened by continuing improvements in the various tests, including harmonisation of methodologies and establishment of standardised protocols.

There are other assessment methodologies that are currently not a part of the assessment system for allergenicity of novel proteins in GM Foods, but have the potential to improve the system when/if they become well enough validated for routine use by regulatory bodies. These include:

- Target serum testing

Target serum testing can be used when specific sera from an individual known to be allergenic to a source is not available or has given a negative cross-reactivity to a novel protein with suspected allergenic potential. The screen involves testing for cross-reactivity with a panel of serum samples that contain high levels of IgE antibodies with a specificity that is broadly related to the source of the transgene. Sources are broadly grouped into yeasts/moulds, monocots, dicots, invertebrates, vertebrates and others. A panel of 50 serum samples with high levels of IgEs to allergens in the relevant group is used to search for IgEs that cross-react with the novel protein. It should be noted that if a transgene was obtained from a bacterial source target serum testing would not be possible as no population of individuals are known to be sensitised to bacterial proteins. Target serum testing to determine the allergenic potential of a protein in fact only detects the ability of that protein to elicit a response in an individual known to be sensitised to another allergen i.e. the novel protein is cross-reactive with a known allergen. This test does not directly address the sensitising properties of the novel protein, but rather its potential allergy-eliciting properties in a previously sensitised individual. It has also been shown recently that the presence of IgE antibodies cross-reactive with an allergen does not always correlate with the development of clinical allergy responses in an individual. Further validation of serum testing methodologies is needed before this can become part of routine assessment of allergenic potential of a novel protein.

- Animal testing models for allergenic assessment of novel proteins

The first animal model for testing for allergenicity of GM foods has recently been developed by researchers at Michigan State University. Funds have been supplied by the US Environmental Protection Agency for this mouse model to be validated. Such a system would greatly enhance the safety assessment of GM food.

Sources:

Taylor, S. (2006) Review of the development of methodology for evaluating the human allergenic potential of novel proteins. *Mol. Nutr. Food Res.* 50: 604-609.

Additional and background information from:

van Ree, R., Vieths, S. and Poulsen, L.K. (2006). Allergen-specific IgE testing in the diagnosis of food allergy and the event of a positive match in the bioinformatics search. *Mol. Nutr. Food Res.* 50: 645-654.

‘Will GE foods cause allergic reactions? Michigan State University scientist receive EPA grant to find out’. [www.checkbiotech.org](http://www.checkbiotech.org). 1 Oct 2006.

## 2.3 GM ADVENTITIOUS PRESENCE

### 2.3.1 GM Rice LLRICE601

During the reporting period long grain rice shipments from the US were found to have adventitious contamination with an unauthorised GM rice line LLRICE601. This resulted in the EU taking an initiative to regulate shipments of rice from the US and seek comment from EU members on the safety of this GM food line.

In November 2006 the Advisory Committee on Novel Foods and Processes (ACNFP), a non-statutory, independent body of scientific experts that advises the UK Food Standards Agency, released a Committee Paper For Discussion: '[ACNFP/79/14 Unauthorised presence of GM rice \(LLRICE601\) in long grain rice from the USA](#)'. Information relating to LLRICE601 and the issue of contamination of US long-grain rice exports is taken from this paper and summarised in this report.

#### 2.3.1.1 Background to issue

- Two types of GM rice have been officially approved for commercialisation and use in feed and food in the US. Developed by Bayer CropScience, LLRICE62 and LLRICE06 are both engineered for herbicide-tolerance. Whilst these authorisations are in place, no GM rice has yet been grown commercially in the US. Rice LLRICE62 is currently being evaluated for use in the EU, and an application has also been received by FSANZ to approve food derived from this GM rice line (an Initial Assessment Report was released by FSANZ on 13 December 2006).
- On 31 July 2006 Bayer CropScience notified the US authorities that 'traces' of another GM rice line, LLRICE601, had been identified in samples of commercial long-grain rice and may have entered the food and feed supply. This rice line was used in field trials in 1998-2001 but was not taken forward for authorisation in the US. LLRICE 601 is also engineered for herbicide-tolerance, and contains the same inserted gene as the other two US authorised rice lines, LLRICE62 and LLRICE06.
- Bayer CropScience does not supply rice seed commercially in the US and the origin of the GM contamination was not identified.
- On 18 August 2006 the USDA officially issued a statement indicating that traces of the GM rice line LLRICE601 had been identified in rice samples. They considered that this GM rice line posed no food or animal feed safety concerns. Levels of adventitious contamination of long-grain rice exports was indicated to be at <0.1%.

#### 2.3.1.2 Background to GM rice line LLRICE601

GM rice line LLRICE601 was developed by Bayer CropScience (formally Aventis CropScience until acquisition by Bayer AG in 2002). The line is engineered for tolerance to the herbicide glufosinate ammonium and was generated as one of a number of GM events

generically named LibertyLink® due to their tolerance to the proprietary glufosinate herbicide Liberty Herbicide. Generation of LLRICE601 was by *Agrobacterium*-mediated introduction of the *bar* gene under control of the Cauliflower Mosaic Virus 35S promoter element. This promoter element confers constitutive expression of the introduced gene, that is, expression in all plant parts at all times. Introduction of the *bar* gene results in production of the enzyme phosphinothricin acetyltransferase (PAT), which confers resistance to the herbicide glufosinate. As resistance to glufosinate was used to select for transformants, no other marker genes were inserted into the line.

The inserted gene cassette in LLRICE601 is very similar to that inserted into the GM rice lines LLRICE62 and LLRICE06. All contain the 35S promoter element fused to the *bar* gene from the bacterium *Streptomyces hygroscopicus*. The main difference in the inserted gene constructs is the transcription termination sequence used. LLRICE601 utilises the 3' untranslated end of the nopaline synthase gene from *Agrobacterium tumefaciens*, while LLRICE62 and LLRICE06 contain the Cauliflower Mosaic Virus 35S termination sequence T35S.

Other differences between unauthorised LLRICE601 and the US authorised lines LLRICE62 and LLRICE06 relate to how the lines were generated. Each line was generated from a different parent cultivar of *Oryza sativa* L. The other main difference was that line LLRICE601 was generated via *Agrobacterium*-mediated transformation while LLRICE62 and LLRICE06 were generated by direct gene transfer.

Rice line LLRICE601 was assessed in small field plots in the US and Puerto Rico from 1998-2001. Inheritance of the trait for resistance to glufosinate enabled selection of a homozygous line containing a single locus insertion. Further selection of this line was carried out for agronomic characteristics and assessment for agronomic equivalence with commercial rice lines was undertaken.

In August 2006, as a result of the identification of adventitious contamination of commercial US rice shipments with LLRICE601, Bayer CropScience applied to the USDA with a request for approval for non-regulated status for LLRICE601. This application was in the form of an Extension Petition to the Approved Petition of non-regulated status for rice lines LLRICE62 and LLRICE06, and provided information on LLRICE601 to justify why its inclusion on the approval should be granted. The USDA subsequently granted approval for de-regulation of LLRICE601 in November 2006.

### 2.3.1.3 *International response to adventitious contamination of US rice shipments with LLRICE601*

- Adventitious contamination of US exports of long-grain rice was considered to be an international issue involving countries in the EU as well as any other countries importing long-grain rice from the US. The European Commission took the lead and approached the US authorities and Bayer CropScience for further information. The extent of the contamination of US exports was unclear and these agencies were unable to guarantee that future rice shipments from the US would not contain the unauthorised GM rice line LLRICE601.

- On 23 August 2006 the European Commission adopted an emergency measure requiring all shipments of US long-grain rice to be tested and certified free of LLRICE601 prior to import into the EU. Member States were charged with the responsibility of controlling imports at their borders and ensuring no contaminated shipments entered the EU. Member States were also required to implement random sampling and analysis of food products already on the shelves.
- EU and US agencies were unable to agree on a common sampling and testing protocol that would ensure a high level of consistency and accuracy of tests. Ongoing issues of contamination in shipments certified as free of LLRICE601 resulted in the European Commission implementing a policy of counter-testing of all imports of US long-grain rice in October 2006. Under this directive, all consignments of US long-grain rice are to be tested at the point of entry into the EU to confirm absence of LLRICE601, and as recently notified by the French, LLRICE62.
- Testing is the responsibility of EU Member State authorities at point-of-entry and is to be undertaken according to EU testing protocols. Testing methods to detect LLRICE 601 and LLRICE62 events were provided by Bayer CropScience and evaluated by the EU Community Reference Laboratory (CRL) at the Joint Research Centre in Italy. The PCR test detects the 35S::*bar* sequences inserted into the rice lines. The CRL noted that the method provided also gave positive results for some other GM crop lines that contain 35S::*bar* sequences, including the EU approved corn line Bt176. This observation highlights the need for comprehensive controls when using this protocol to detect LLRICE601 in complex food or feed matrices. Subsequently a test for sequences specific to LLRICE601 was provided by Bayer CropScience and was confirmed by the CRL not to detect other GM events. Details of the testing methods and the CRL's reports on the methods can be found at <http://gmo-crl.jrc.it/LLRice601update.htm>.
- The European Commission passed the information provided by Bayer CropScience on LLRICE601 to the EFSA to obtain advice on the safety of this GM rice line. On 15 September 2006 the EFSA's GMO Panel responded to the European Commission. In formulating its response the panel took into consideration the scientific data on LLRICE601 provided by Bayer CropScience, data from risk assessments carried out by the US authorities, existing scientific data on the similar GM rice line LLRICE62 (EFSA is in the process of carrying out a risk assessment for approval of LLRICE62 for food and feed import and processing) and similarity of the introduced protein in LLRICE601 to that in other approved GM foods such as corn lines Bt11 and T25. The Panel's response stated that while insufficient data was available for them to carry out a full safety risk assessment of rice line LLRICE601, based on the available data "the consumption of imported long-grain rice containing trace levels of LLRICE601 is not likely to pose an imminent safety concern to humans or animals". EU member states such as the UK used this response as the basis for advice to consumers that the consumption of US long-grain rice that they had at home was deemed to be safe. Food retailers were however advised of their responsibilities under the regulations for GM foods and encouraged to ensure that the products on their shelves remained legal, and that any products known to contain LLRICE601 be removed from sale.

- The UKFSA carried out a survey of rice shipments held at UK rice mills. The aim of the survey was to ensure that no further contaminated shipments were able to enter the UK food market. A total of 31 samples of US long-grain rice were taken from 8 mills in the UK during three weeks in September/October 2006. Of the 31 samples tested, 3 were positive for the presence of rice line LLRICE601, to a Limit of Detection of 0.02%. Positive results were notified to the mills from which the samples were obtained, and to local authorities. It is significant to note that one mill owner had previously had the same batch of US long-grain rice as sampled by the FSA tested by a certified laboratory and the result had been non-detection of LLRICE601. This is likely reflective of the very low level of adventitious contamination of rice shipments and the Limits of Detection of testing methods from different testing agencies. The FSA report on the testing was released in November 2006 (see <http://food.gov.uk/science/surveillance/>).

#### *2.3.1.4 Issues highlighted by the contamination of US long-grain rice exports with LLRICE601*

The adventitious presence of GM materials in global imports/exports is an ongoing issue, highlighted during this reporting period by the contamination of US long-grain rice exports with the unapproved GM line LLRICE601. This contaminating GM rice line was unapproved in both the country of export (the US) and import countries in the EU, and potentially Australia and New Zealand. Similar issues could arise when a GM line has been approved in one country but not another, as with rice line LLRICE62 detected in US rice shipments to France. This adventitious contamination presents a number of difficulties to regulatory agencies:

- Under EU and FSANZ regulations, unauthorised GM material in food is illegal at any level. Detection of adventitious contamination with such material requires specific and highly sensitive testing protocols. Where contamination is from an unapproved GM line, such as LLRICE601, such tests may not be readily available until after a contamination event has occurred and delays may then be encountered in validation of tests.
- Testing protocols for rice line LLRICE601 highlighted the increasing difficulty in obtaining event specific tests for particular GM crop lines, as the same foreign gene insertion constructs are being introduced into a number of different plant species. Where adventitious contamination with unapproved lines is being tested for, it is necessary to be able to distinguish between the unapproved line and any other approved GM crop that may be present, either as a result of impurities in seed consignments, or due to more than one ingredient being used in a complex food matrix.
- Limits of Detection for GM testing protocols can vary between testing agencies, and may result in false negative tests when adventitious contamination is at very low levels. This highlights the need for testing bodies to implement standardised testing methodologies. Problems may be encountered in testing body disclosure of methodologies due to commercial competition.
- The present guidelines for assessment of GM food safety are intended to ensure public safety when the material is marketed and consumed on a permanent basis. Full risk assessment of the GM line is undertaken as a part of the approval process of a particular country or approval body (e.g: the EU). GM lines are usually unapproved as an approval has not been sort, rather than them having failed to gain an approval due to safety issues.

Therefore, the data required to fully assess the safety of an unapproved GM line that may adventitiously contaminate the food chain are often not readily available.

The adventitious presence of GM material was one of the issues discussed at the 6<sup>th</sup> Session of the CODEX *ad hoc* Intergovernmental Task Force on Foods Derived from Biotechnology, held in Japan in late November 2006. The Task Force agreed on a project document for future work: ‘Annex to the Guideline for the Conduct of Food Safety Assessment of Foods Derived from Recombinant-DNA Plants or Low-level Presence of Recombinant-DNA Plant Material’ and agreed to forward the project document to the Executive Committee for critical review and for approval by the next Session of the Commission in July 2007. It is hoped that some of the issues discussed above will be addressed in this international forum.

Sources:

[ACNFP/79/14 Unauthorised presence of GM rice \(LLRICE601\) in long grain rice from the USA](http://www.food.gov.uk/multimedia/pdfs/acnfp_79_14.pdf)  
[http://www.food.gov.uk/multimedia/pdfs/acnfp\\_79\\_14.pdf](http://www.food.gov.uk/multimedia/pdfs/acnfp_79_14.pdf)

Additional background information from:

UK Food Standards Agency website at <http://www.food.gov.uk>  
European Commission website at [http://ec.europa.eu/food/food/biotechnology/index\\_en.htm](http://ec.europa.eu/food/food/biotechnology/index_en.htm)  
Codex website at <http://www.codexalimentarius.net/web/archives.jsp?lang=en>

### **3 PART B: FOODS FROM CLONED ANIMALS**

This section presents recent international information on the safety of foods from cloned animals. Issues associated with transgenic animals as foods are not covered in this report.

#### **3.1 INTRODUCTION TO CLONING OF FOOD ANIMALS**

Assisted reproductive technologies (ARTs) have been used in animal production systems for over a century. One of the more recent developments within this area is nuclear transfer technology (NTT), or more colloquially ‘cloning’. Nuclear transfer techniques can be divided into two types:

- i. Embryonic nuclear transfer (ENT), where the nucleus from a very early embryo (blastocyte) is taken and transferred into the cytoplasm of a recipient cell that has had its own nucleus removed (e-nucleated host cell). The blastocyte stage from which the nucleus is taken is prior to morphologically distinct differentiation of cell types in the embryo and directs the recipient cell on to develop into an embryo.
- ii. Somatic cell nuclear transfer (SCNT), where a differentiated animal cell nucleus is transferred into an e-nucleated recipient cell. In this system the nucleus from the partially or terminally differentiated cell re-programmes the e-nucleus back to a non-differentiated state, which then directs development of the cell into an embryo.

Both of these systems have been used to successfully generate cloned animals. Much of the research in animal cloning is still at a developmental stage and issues have been seen with an increased frequency of anomalies in offspring compared to those from conventional breeding systems. The types of anomalies seen are, however, consistent with those resulting from the use of other ARTs such as *in vitro* fertilization. While currently a time consuming and expensive technology to implement ‘cloning’ does have potential to enhance agricultural practise, as elite animals are likely to be used as nuclear donors for the increased production of desirable characteristics.

#### **3.2 SAFETY OF FOOD FROM CLONED ANIMALS**

##### ***3.2.1 FDA releases draft documents on the safety of food from cloned animals***

In December 2006 the USFDA released three documents on the safety issues associated with animal cloning – a Draft Risk Assessment (DRA), a Proposed Risk Management Plan and a Draft Guidance for Industry. While the cloning of animals has not been illegal in the US, there has been a voluntary moratorium on the selling of milk or meat from such animals, requested by the FDA to allow it to study the safety aspects of the issue. No other countries have yet approved food from cloned livestock, although a number are considering it and are likely to base their recommendations on information in the FDA assessment. The FDA is currently seeking comment from the public on the three documents before releasing a final assessment.

3.2.1.1 *A Risk-Based Approach to Evaluate Animal Clones and Their Progeny – Draft*  
<http://www.fda.gov/cvm/CloneRiskAssessment.htm>

Background to development of the Draft Risk Assessment

The USFDA has been in consultation with clone producers and relevant industries since the late 1990s with respect to potential safety and regulatory implication of the use of cloning technologies. In 2000 the National Academy of Sciences (NAS) was asked to perform an independent scientific review of the available data on the safety of cloning. In 2001 the FDA requested that clone producers not introduce meat or milk from clones or their progeny into the food market until the NAS report had been completed. The NAS issued their report in 2002. The report concluded that there is “no evidence that food products derived from adult somatic cell clones or their progeny pose a hazard (i.e. there is no evidence that they present a food safety concern)”. The report did however note that at that time there was no published data comparing the composition of meat or milk from clones with meat and milk from conventional animals.

Based on the limited data from which the NAS was able to draw its conclusions, the FDA subsequently carried out an independent analysis of all of the data relevant to assessing the health of clones and their progeny and to assessing risks associated with consumption of edible products from these animals. The FDA stresses that the resulting Draft Risk Assessment is a framework by which science-based questions regarding animal health and food consumption risks are evaluated. Recommendations for managing those risks and circumstances under which foods from cloned animals and their progeny may be released for commercial use are contained in the accompanying Proposed Risk Management Plan and Draft Guidance for Industry. It is also stressed that the Draft Risk Assessment does not address questions associated with the ethics of cloning.

The following section summarises issues addressed in the almost 700 page Draft Risk Assessment (DRA) and accompanying documents. Emphasis is placed on sections in the DRA that focus on risk associated with consumption of food from cloned animals as this is of direct relevance to the NZFSA. Issues relating to the health of cloned animals themselves that are not associated with risks for food consumption are not addressed.

Risk Assessment Methodology

The DRA is the result of a qualitative analysis that identifies and characterises the hazards that may result from animal cloning. These hazards are assessed in the context of other ARTs, for example artificial insemination, that are currently in use in the animal production industry. A finding of “no additional risk” applied to the safety of food products from cloned animals implies no additional risk relative to corresponding products from conventional animals, including those produced using other ARTs. The goal of the DRA was to determine whether any unique hazards arise from cloning technologies that are not noted in comparators, or have not been identified in cattle, swine, sheep or goats produced using other ARTs.

Food Consumption Risks

Unlike transgenic animals, cloned animals have no introduced 'foreign' DNA in their genome. Their genetic relationship to the parent organism is closely analogous to a twin, albeit born at a different time. As no genetic material has been introduced into the animal via genetic engineering, any food consumption risk from a cloned animal is assumed to be likely the result of a mis-programming of naturally occurring genes within the genome of the clone. Such epigenetic mis-programming, often related to changes in the methylation status of the genome, can occur in offspring of 'normal' sexual reproduction and has been noted in the offspring obtained from other ARTs currently in use. Subtle hazards and potential risks to consumption from animal clones were therefore considered in the context of other mutations and genetic re-programming that can occur in all food animal populations.

A two-pronged approach was taken to determine whether epigenetically induced changes in the expression of a clone's genetic material could result in subtle food hazards that pose a food consumption risk.

- i. Critical Biological Systems Approach: This involved a systematic review of the health of the animal clones and their progeny. The premise is that a healthy animal is likely to produce safe food products, and therefore conversely, that animals identified with abnormalities are likely to be considered unsuitable for foods. This type of assessment is utilised with food products of conventional animal origin in order to avoid the consequences of a range of animal health issues. Within the assessment the emphasis was on potential for subtle hazards and so the animal health data were evaluated to a fine level of resolution – often to the level of individual animals. Adverse outcomes in animal clones were evaluated to provide an insight into identifying food consumption risks. Health risks to the animals themselves were addressed in other sections of the DRA not reported on here.
- ii. Compositional Analysis Method: This assessment assumes that food products from healthy animal clones and their progeny, that are not compositionally different from the corresponding product from conventional animals, pose no additional risks for consumption.

Conclusions within the DRA were broken down into animal clone type:

- Cattle clones: Edible products from juvenile and adult cattle clones were determined to pose no additional food consumption risks relative to corresponding products from contemporary conventional comparators. It was however concluded that food products from perinatal bovine clones (clones at 5 months before and up to 1 month after birth) may pose some very limited human food consumption risks. It is during this period that cattle clones in particular demonstrate symptoms of fragility that may be the result of epigenetic mis-programming and this period is when unexpected clone death is most likely to occur. However, it is stated that clones that have been shown to be 'fragile' during this period and survive then tend to develop normally and have not been shown to differ significantly to conventional newborns in key physiological measurements. It is concluded that whilst possible it is unlikely that food consumption risks have been introduced into these animals.
- Swine clones: Data for this assessment was weighted towards adult, market-sized animals and as with adult bovine clones the conclusion was that edible products from swine clones pose no additional food consumption risks relative to corresponding products from contemporary conventional comparators.

- **Sheep clones:** Insufficient information was available for conclusions to be drawn relating to the food consumption risks for sheep clones. The data available to the FDA was related to anomalies in fetal sheep clones that had died or been terminated, or from pathological studies on sheep clones that had died prematurely. Whilst it was acknowledged that this information is valuable for understanding molecular and developmental issues associated with the cloning technology, the data are not relevant to determining food consumption risks as the animals involved would not have been allowed to enter the food chain based on general health parameters. No reports were obtained that addressed the composition of meat or milk from sheep clones.
- **Goat clones:** Whilst no meat or milk compositional data was available for goat clones, the FDA concluded from the other available data that edible products from goat clones pose no additional food consumption risks relative to corresponding products from contemporary conventional comparators.
- **Clone progeny.** Due to the high cost of producing primary cloned animals, it is likely that the majority of clone-derived food in the market place will be from the progeny of clones. Clone progeny are the product of sexual reproduction and risk assessment for food products derived from clone progeny is therefore relative to consumption of similar products from conventional, sexually derived, animals. This is based on an underlying biological assumption that the process of germ cell development in sexual reproduction ‘re-sets’ any epigenetic re-programming of genome expression that may have occurred in the parent. This assumption has been demonstrated in a mouse model system whereby phenotypic alternations in parent clones were not passed on to their sexually derived offspring. Studies on the health and meat composition of clone progeny provided no evidence for additional food consumption risk from clone progeny, compared to comparable progeny from non-clone animals.

**Overall the DRA concludes that “no adverse outcomes have been noted in clones that have not been observed in animals derived via other ARTs or natural mating that already enter the food chain unimpeded”, and that clone progeny pose no additional food consumption risk. This is in agreement with the conclusions from the NAS 2002 preliminary finding on the safety of food from cloned animals.**

*3.2.1.2 Animal Cloning: Proposed Risk Management Plan for Clones and Their Progeny*  
[http://www.fda.gov/cvm/CloningRA\\_ProposedPlan.htm](http://www.fda.gov/cvm/CloningRA_ProposedPlan.htm)

Risk management is a set of activities that integrates information from a risk assessment with other information in order to make decisions about the need for and level of risk mediation required. The Proposed Risk Management Plan released by the FDA in conjunction with the DRA on the safety of cloned animals is designed to identify the relevant issues and to present proposed actions required to manage the risk(s) identified in the DRA.

There are acknowledged uncertainties associated with the assessment in the DRA. Uncertainties are a factor of any risk assessment and the degree of uncertainty therefore contributes to the level of risk determined. With respect to food consumption-related issues the Proposed Risk Management Plan (PRMP) largely deals with the management of these uncertainties. These uncertainties fall into three categories:

- i. Empirical Data: The degree of confidence that can be placed on conclusions arising from the assessment of empirical data sets is related to the size of the data sets. Confidence is generally greater in conclusions from large data sets than from small or incomplete data sets. The use of a Critical Biological Systems Approach in the DRA was designed to allow adjustment for small studies and incomplete data sets by allowing evaluation of all available data regardless of source and subject in order to determine whether common anomalies could be detected. As a part of the PRMP the FDA intends to review any new data that becomes available during the public comment period and include this in the Final Risk Assessment.
- ii. Biological Assumptions: Uncertainties in the risk assessment can be a product of the assumptions made about biological systems involved in the generation of clones. In particular with respect to the mechanisms of epigenetic re-programming of genome expression in clones. Scientific understanding of these issues is still imperfect and research continues to provide information about the molecular mechanisms underlying these processes. As an acknowledgement of this biological uncertainty the PRMP indicates that the FDA will continue to monitor the scientific information from this expanding field to ensure that the positions held as they apply to clones remain appropriate.
- iii. Technology Changes: The DRA evaluated clones themselves rather than the methodologies utilised to generate them, however, it is acknowledged that most of the clones considered in the DRA were generated using relatively similar methods. Major changes in the technology used to produce clones could result in the introduction of uncertainties with respect to risks for food consumption. Similarly application of new methodologies to produce clones from food-producing species not considered in the DRA might also introduce uncertainty. To address this possibility the FDA intends to continue to monitor the technology and the science that underpins it in order to determine if technology changes may impact on the conclusions drawn in the DRA.

As more clones are generated and more data collected the degree of uncertainty surrounding the risk of clones for food consumption should decrease. To date much of the data on clones have related to the development of the technology and have involved studies on fetal and young animals. There is likely to be less uncertainty about the health of clones as they age and have more time to exhibit the full range of functions expected of breeding stock. Similarly as more clones are allowed to reach maturity more information on composition of meat and milk products and on clone progeny can be obtained.

The PRMP is therefore designed to accommodate the ongoing assessment of available data to underpin conclusions drawn in the DRA, with respect to risks to food consumption, and details the specific intentions of the FDA to:

- i. *Monitor and review additional animal health and food composition data on animal clones or their progeny as they become available.*
- ii. *Monitor and review changes in animal cloning techniques and technologies.*
- iii. *Continue to consult with clone producers to review changes in the technology.*

- iv. *Monitor and maintain knowledge base on the biology of epigenetic mechanisms governing gene expression and their role in nuclear transfer.*

**3.2.1.3 *Guidance for Industry Use of Edible Products from Animal Clones or Their Progeny for Human Food or Animal Feed***

<http://www.fda.gov/cvm/Guidance/guideline179.htm>

The Draft Guidance Document describes the FDA's recommendations to industry with respect to the introduction of edible products from animal clones and their progeny into the human food supply. The basis of recommendations is that various systems are already in place in the US to ensure the safety of human food derived from animal products. These systems do not require that information be provided on how animals were generated (e.g. natural mating versus ARTs such as artificial insemination).

As the DRA concluded that no unique hazards were identified for foods from cloned animals or their progeny, such foods would be subject to the same food safety systems as food from any other animal. Specifically, it was concluded that there is no scientific basis for the recommendation of any additional safeguards related to the use of products from cattle, swine or goat clones or their progeny. As the DRA was not able to draw conclusions on the safety of foods from sheep clones, due to insufficient data, the FDA recommends that edible products from sheep clones not be introduced into the human food market at this time.

**3.2.2 *Summary of FDA DRA and associated documents and implications for legislation on food from cloned animals***

The FDA's Draft Risk Assessment for animal clones and their progeny, and the associated Risk Management Plan and Guidance of Industry documents are the result of a four year analysis of the available scientific literature and a comprehensive examination of the health of livestock clones. During this time the FDA requested a voluntary moratorium on industry for the release of food products from cloned animals into the human food market. The conclusions from the DRA were that there is no scientific evidence to indicate any unique food consumption hazards from cloned animals or their progeny compared to conventionally bred animals or animals derived from other ARTs. The resulting Guidance for Industry documents reflect this conclusion with a recommendation that no additional safeguards are necessary to ensure the safety of food products from cloned animals or their progeny other than those that would be applied to food products from any other animal.

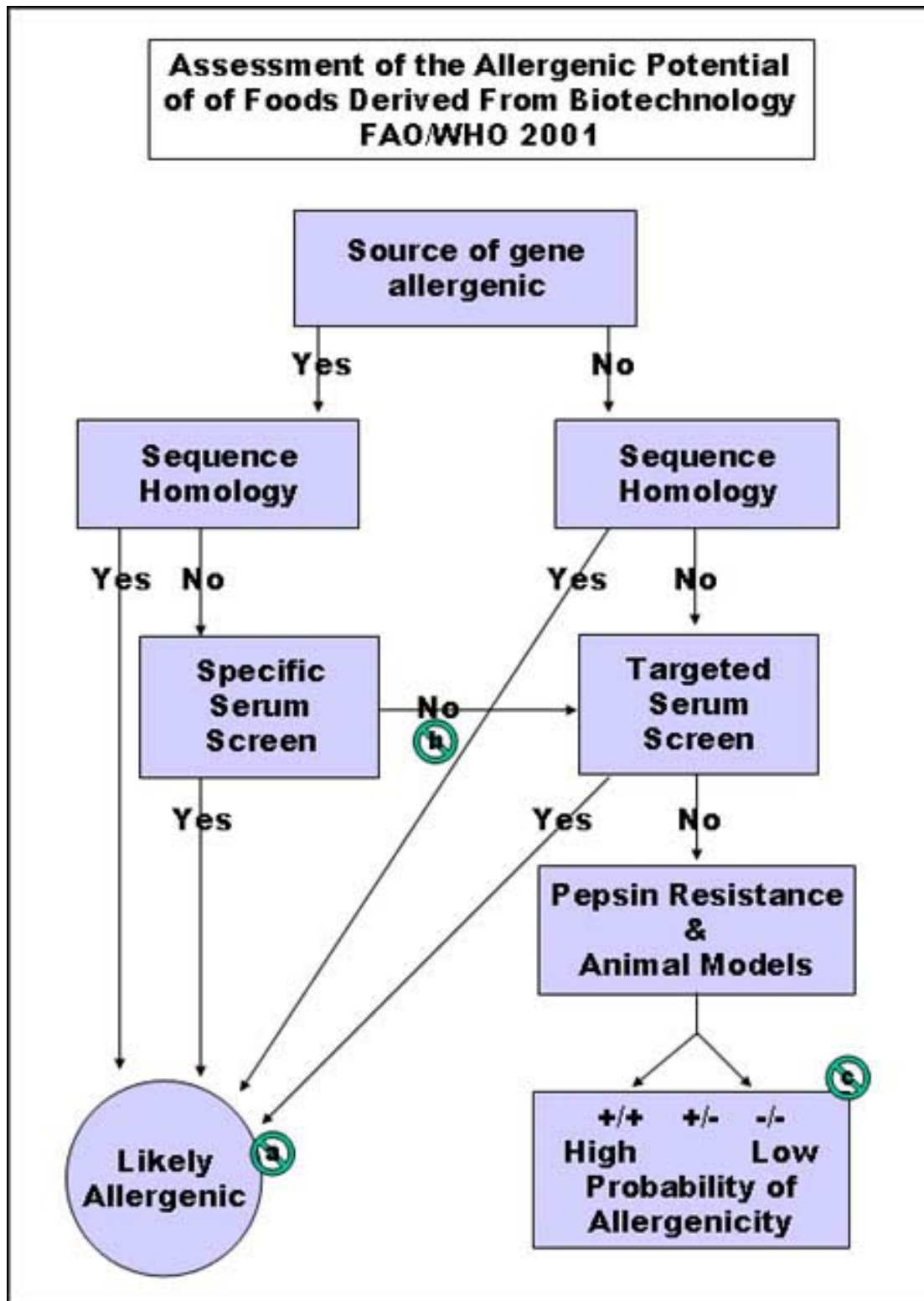
The Risk Management Plan, however, acknowledges that there are uncertainties associated with the DRA. Risk management concentrates on the ongoing monitoring of scientific information on animal clones and the technologies used to generate them. The FDA intends to continue to interact with scientific and professional bodies involved in generation of animal clones as well as industry parties and acknowledges the issue of public concern in the area of animal cloning ethics, and safety of food from animal clones. As the released document are the beginning of an interaction with the public on food safety issues the FDA has asked producers of clones, and livestock breeder, to continue the voluntary moratorium

on introduction of edible products from clones into the food market until public submissions on the documents can be considered.

The release of the DRA and associated documents by the FDA is likely to impact on global decisions regarding the safety of food from cloned animals. As yet no such food has been formally approved for release in any country. This is partly a reflection of the developmental state of animal cloning in many countries. Generation of animal clones continues to be an expensive enterprise and production of primary clones for the food market is likely to be some way off for most commercial producers. However, as use of the technology increases and more animals are produced and taken through to adulthood the potential for food from the progeny of clones to be commercialised is likely to increase. An example of this was the announcement, in late December 2006, that an offspring from an American beef clone had been born on a British farm. Whilst the pedigree of the calf is of prime importance to the owner, it is possible that its milk may be mixed with milk from other animals on the farm and introduced into the human food market. At the end of the reporting period the UKFSA and the European Commission were considering how to respond to such events and were awaiting the release of the FDA documents on which they intend to base their decisions.

The FDA documents do not consider the safety of food from transgenic animals. However, it is noted that transgenesis and cloning are often implemented together in order to obtain an elite animal. Transgenic animal research is still very much in the developmental stages and it is unlikely that any transgenic cloned animal products would enter the human food market in the near future. However, it is important to emphasise that a combination of transgenesis and cloning introduces different issues into a risk assessment than for cloning alone. The ability to distinguish whether any abnormalities in the development of a transgenic clone are related to the insertion of foreign DNA and/or epigenetic re-programming of genome expression unrelated to the inserted DNA is technically more demanding than for cloning technology. The approval of food products from transgenic animal clones should be assessed under different criteria than that for clones alone and on a case-by-case basis as is currently required for the approval of food from genetically modified crops.

## ANNEX A FAO/WHO 2001 DECISION TREE



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