



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

July – December 2007

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AND FOOD FROM CLONED ANIMALS

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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 2007, and includes:

1. *Mini-chromosomes in the generation of transgenic plants.*

Two different methods to engineer mini-chromosomes in plants and to utilize them as platforms for the insertion of 'foreign' genes are described. Mini-chromosomes have the advantage of enabling the rapid development of transgenic lines with multiple transgenes (so called 'stacked' traits). It is suggested that they could be utilized in the future to engineer plants with complete transgenic metabolic pathways. As mini-chromosomes contain all introduced elements as a single discreet unit, separate from the parental genome, they are less likely to cause changes to the parental phenotype due to insertion/disruption events and can be manipulated as a unit in predictable inheritance breeding. Potential implications for regulation and testing of GM foods are discussed.

2. *Use of RNA Interference techniques (RNAi) in biotechnology.*

RNA interference techniques are being increasingly utilized to shut off or down regulate the expression of genes, either endogenous or introduced, in order to engineer metabolic pathways in plants. Such metabolic engineering can be used to alter the nutritional value of plants or to remove toxicants. During the reporting period a review of these techniques was published that included a number of recent examples of their use in engineering of food plants. This review is summarised and implications for the use of these technologies on GM food testing and regulation is provided.

3. *Novel techniques to profile amino acids in GM foods for the assessment of equivalence.*

Safety assessment of foods from GM plants relies predominantly on the concept of substantial equivalence. That is, the demonstration that the transgenic plant is essentially equivalent to its non-GM parent in all but the desired effect of the introduced transgene, and that the desired effect has no unintended effects in the plant. Currently equivalence is assessed by analysing a range of targeted constituents such as nutritional components, antinutrients and toxicants. These methods are limited in their ability to determine unintended effects. A new method has been developed to profile the amino acid content of GM plants with respect to amino acid L/D conformation. It is suggested that the data obtained using this method would add further rigor to the assessment of equivalence.

4. *90-day rat feeding studies revisited.*

In the safety assessment of foods from GM plants the requirement for long duration rodent feeding trials is optional. This relates to differences in opinion within the scientific community on the validity of results obtained from trials carried out using traditional methodology. This included issues in differentiating negative effects that may relate to the GM plant but not to the transgene product *per se*. A new 90-day rat feeding trial design has been published that specifically addresses the need to distinguish safety issues related to effects due to the transgene product from secondary effects of the GM plant in food.

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1. INTRODUCTION

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

The studies/topics have been chosen from within the following subject areas:

- Novel techniques for developing GM plants/animals and the implications on current detection methods;
- Animal feeding studies – specifically within the area of foods derived from GMFs or foods from cloned animals;
- Food safety and/or composition studies on GMFs and/or foods from cloned animals;
- Adventitious presence issues for GMFs and new GM varieties approved for food use, with particular emphasis on describing how other countries have responded with regard to audits and/or testing regimes and safety assessments, and providing relevant information and discussion of the actual food safety risks.

This is the first report for the 2007/2008 year and covers the period from July to December 2007.

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

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

Source material:

Specific studies that are discussed in this report are referenced at the end of the relevant section. Any additional source of background material is also referenced. Where there is no reference to background material it is either taken from the study document or is general scientific knowledge of the report author.

1.1. Abbreviations used throughout this document:

EC: European Commission

EFSA: European Food Safety Authority

EU: European Union

FSANZ: Food Standards Australia New Zealand

OECD: Organisation for Economic Co-operation and Development

2. NOVEL TECHNIQUES FOR DEVELOPING GM PLANTS

2.1. Mini-chromosomes in the generation of transgenic plants

2.1.1. What are chromosomes?

In eukaryotic cells, such as in plants and animals, the hereditary molecule DNA is packaged into structures called chromosomes. These chromosomes are located within the cell nucleus and consist of DNA that is tightly coiled, many times, around support proteins called histones. This allows a large amount of genetic material to be packaged into a small space. Chromosomes have a common general structure (see Fig 1.).

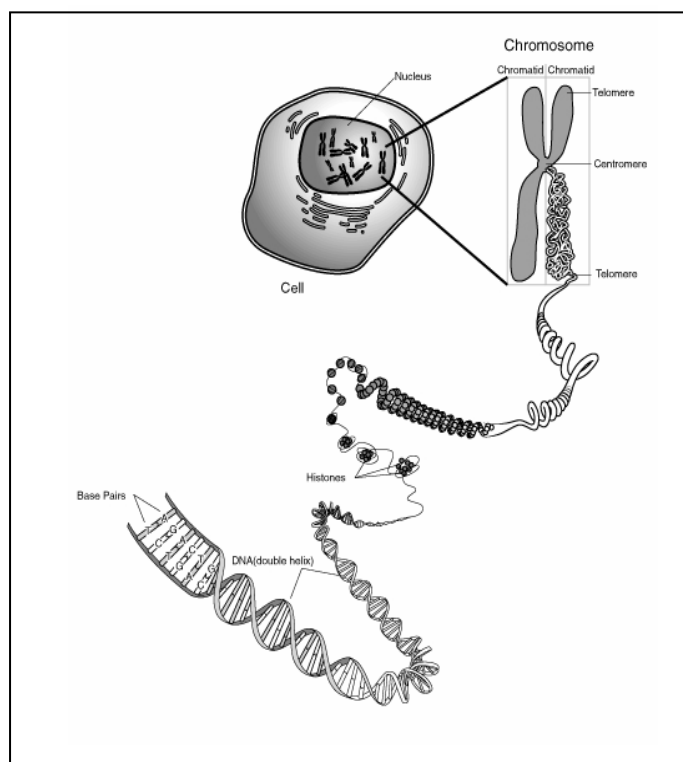


Figure 1 Chromosome structure

Taken from: www.compbio.cs.sfu.ca

This structure includes a central constriction point called the centromere; which divides the chromosome into two sections, or “arms.” The location of the centromere on each chromosome gives the chromosome its characteristic shape and is involved in the process of chromosome replication when cells divide. A telomere region is located at each end of the linear chromosome and protects the chromosome end from instability. The telomeres

consist of tandem arrays of highly conserved DNA sequence and associated proteins. The DNA sequence is usually 5-8 nucleotides in length and is highly conserved among eukaryotic organisms. Individual genes are arranged along the chromosome arms.

Chromosomes are not visible in the cell's nucleus (even with a light microscope) when the cell is not dividing. However, the DNA that makes up chromosomes becomes more tightly packed during cell division and is then visible using a microscope. An example of a complement of condensed chromosomes visible during cell division is shown in Fig 2. As cells divide within an organism e.g. during growth, they copy and transfer their chromosome complement to the daughter cells (see Fig 3.). In this way the genetic blueprint is retained in all cells.

An organism obtains half of its genetic material from each parent, as set of chromosomes (see Fig 4). The number of chromosomes acquired from one parent is known as the haploid number ('n' number). The haploid number is characteristic. For example: corn $n=10$, while wheat $n=21$ and soybean $n=20$. The complement of chromosomes from both parents is the diploid ($2n$) number, e.g. corn $2n=20$. Plants can become more complex as some have the capacity to carry multiple copies of a single chromosome or an entire chromosome set. This is generically known as polyploidy. Alfalfa is an example of a polyploidy plant and has 4 sets of chromosomes ($n=8$, $4n=32$). This ability has been utilized in plant improvement as it can confer such characteristics as increased plant vigour. The similar situation in humans is detrimental – Down's syndrome is the result of 3 copies, rather than 2, of one of the chromosomes in the human complement.

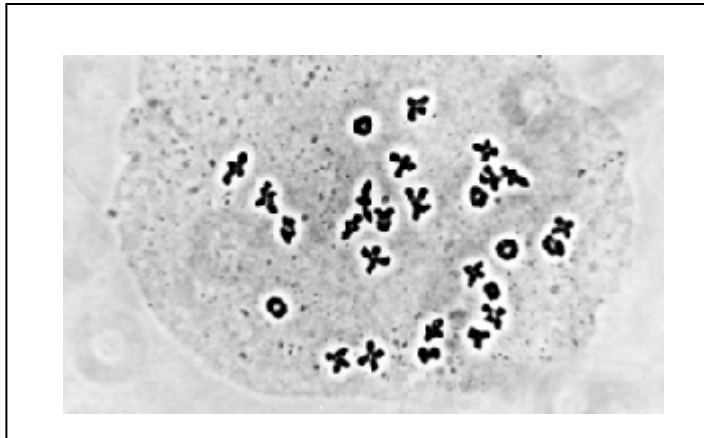


Figure 2 Chromosomes within a cell nucleus

Taken from: www.learn.genetics.utah.edu

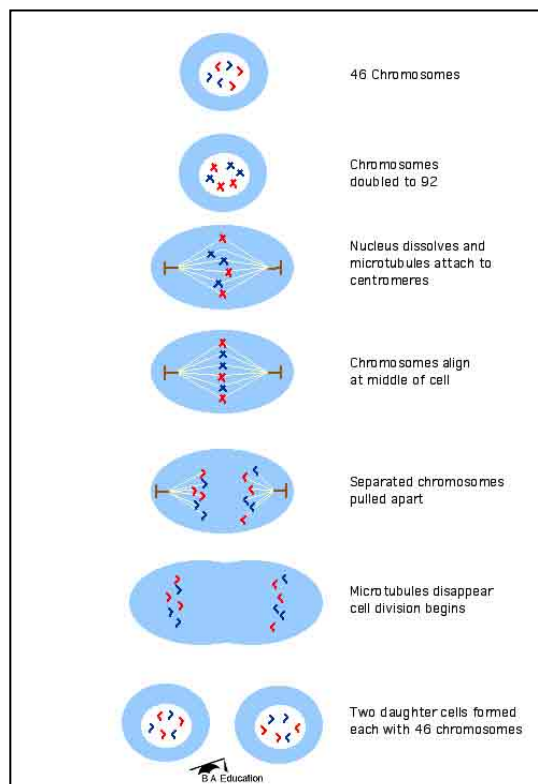


Figure 3 Cell division by mitosis

Taken from: www.ba-education.demon.co.uk

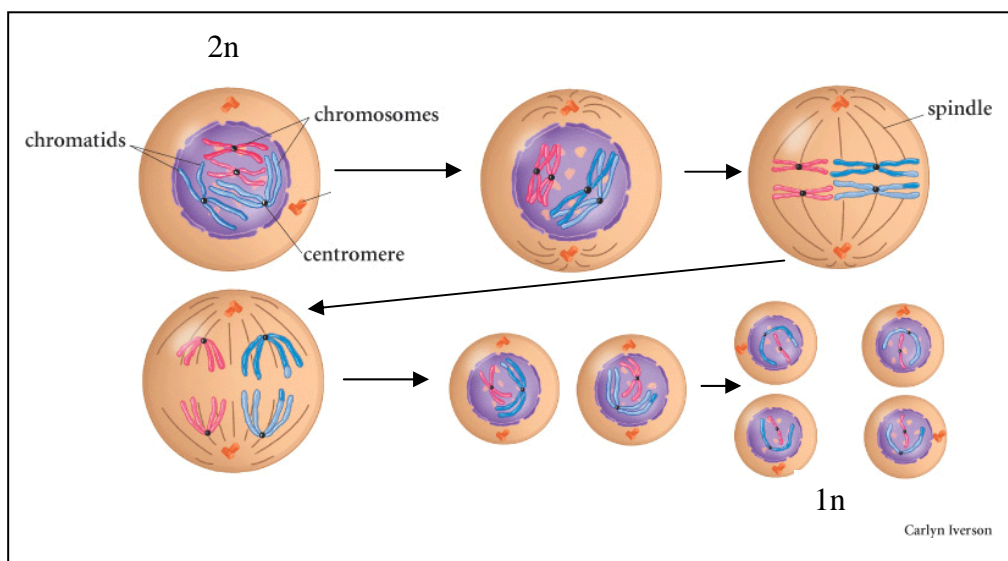


Figure 4 Generation of haploid (1n) ovule/pollen cells by meiosis

Taken from: www.houghtonmifflinbooks.com/booksellers/press_release/studentscience/gif/meiosis1

2.1.2. What are mini-chromosomes?

As outlined above, a chromosome is made up of a centromere region and two telomere regions, separated by a large amount of intervening DNA sequence. This intervening sequence carries the gene complement of the organism as well as regulatory DNA and DNA of unknown function. A mini-chromosome can be engineered to contain a centromere region, with or without telomere regions, and with little else in between. Such 'artificial chromosomes' have been used for some time in yeast systems (Yeast Artificial Chromosomes, YACs, developed during the 1980s) and in human cell systems (developed in 1997) to manipulate and deliver DNA segments containing multiple genes to the cells. These mini-chromosomes are recognised by the cell replication machinery and can be passed on to daughter cells within an organism and through generations to progeny.

Recently mini-chromosomes have been developed to enable the introduction of foreign genes into plants. Mini-chromosome gene transfer systems for corn have been described in two publications released during the reporting period. A summary of the published work, along with implications for plant biotechnology and for the detection and regulation of GM material in food is provided.

2.1.3. Mini-chromosomes engineered in corn via telomere truncations

In May 2007 Weichang Yu and co-workers from University of Missouri, USA, published the results of ongoing work to construct mini-chromosomes in corn using a telomere truncation method. This system modifies natural chromosomes found within the corn. *Agrobacterium*-mediated transformation was used to introduce a segment of DNA into corn cells that contained a selectable marker for herbicide resistance, along with an array of repeat telomere sequences. Introduction of this DNA segment resulted in fracture of existing chromosomes at the site into which the telomere sequence inserted. This caused a truncation of the chromosome, but re-established a telomere sequence at the new end, and thus, potentially, a truncated but functional chromosome. However, due to the deletion of large amounts of genetic material from chromosomes with large deletions, recovery of these chromosomes through the genetic transformation process was difficult. It is also unlikely that such chromosomes could be passed to offspring of a diploid parent as the lack of essential genes would make haploid ovule or pollen cells unviable. Yu *et al.* did, however, recover a plant carrying a truncated version of corn chromosome 7, but the plant was a spontaneously generated tetraploid individual (i.e. contained 4 copies of the haploid 'n' chromosome number). The truncated chromosome was able to be retained because essential genes that had been deleted in the truncation process were provided by the duplicate set of chromosomes. Traditional breeding methods were then able to be used to obtain diploid plants that contained this mini-chromosome.

This result demonstrated the ability to engineer mini-chromosomes from the normal 'A' chromosome complement in corn using telomere truncation methods. The frequency of generation was very low, however, and required the use of a tetraploid plant with repeated backcrossing to generate a stable mini-chromosome in a diploid line.

Within corn there are two types of chromosomes. The 'A' chromosomes make up the functional genetic complement of chromosomes. However, there are also supernumerary 'B' chromosomes. These B chromosomes occur in a large number of plants and animals and have been well described in corn. They are non-essential chromosomes that are not necessarily found in all organisms of a population and can be found in varying numbers in

individuals within the population. Because of their non-essential nature B chromosomes were considered a potential alternative target for engineering mini-chromosomes in corn. They have the advantage of being genetically inert and therefore naturally tolerated within a chromosome complement without altering the phenotype of the plant. In addition they have been shown not to recombine with the A chromosome complement so can be maintained separately within the chromosome complement of a plant. To this end Yu *et al.* used a biolistic transformation method¹ to introduce their telomere-repeat DNA construct into corn cells. Using this method they recovered truncated B chromosomes with a much higher frequency than truncated A chromosomes. Of 281 transgenic events only seven showed fragments of A chromosomes, while 45 transgenes were recovered on normal B or truncated B chromosomes. The size of mini-B chromosomes obtained ranged from very small to almost the size of the full sized B chromosome.

The mini-B chromosomes were assessed for their ability to be transmitted from one generation to another and all showed stable transmission. They were never seen to recombine with the A chromosome complement, demonstrating that any transgenes contained on a mini-B chromosome could be transmitted as a unit. Interestingly these mini-chromosomes contained the herbicide resistance gene (*bar*) and a visible chromogenic marker gene (beta-glucuronidase, *gus*) from the inserted DNA construct, and the plants showed an herbicide resistant phenotype and GUS staining was visible. This demonstrated that while normal corn B chromosomes are genetically inert the engineered mini-B chromosomes that contained introduced gene constructs were able to express these constructs in a normal manner. This suggests that the lack of gene activity on normal B chromosomes is not due to a generic suppression of transcription, and thus mini-B chromosomes can be expected to be able to express 'foreign' gene constructs in plants.

Another advantage to targeting B chromosomes for engineering mini-chromosomes is that mini-B chromosomes can be manipulated during plant reproduction, by crossing with plants with or without B chromosomes, to create dosage effects. Male parents were shown to be able to transmit up to four copies of a mini-B chromosome to an offspring, which along with

¹ See Section 2.1.1 of current Awareness report FW0771 for a description of biolistic transformation.

one copy from a female parent could enable up to five copies of a transgene to be expressed in a plant line. This offers the potential for higher rates of production of transgene products.

Yu *et al.* also demonstrated that additional transgenes could be added to a mini-B chromosome by incorporating a site specific recombination system into the DNA construct inserted into the mini-B chromosome. They utilized a Cre/lox recombination system² to show that they could move transgene DNA from the chromosome of one parent to the mini-B chromosome from the other parent in the hybrid offspring. This system was not highly efficient nor was the recombination event transmitted to progeny of a self-cross, suggesting a non-uniform distribution of recombination events in the cells of the hybrids, in particular an absence in the developing ovule/pollen cells. Nevertheless, this result did demonstrate the potential to use site-specific recombination systems to add or delete transgenes from a mini-B chromosome platform.

Sources:

Yu, W., Han, F., Gao, Z., Vega, J.M. and Birchler, J.A. (2007). Construction and behaviour of engineered minichromosomes in maize. *PNAS (USA)* 104 (21): 8924-8929.

Background:

Yu, W. and Birchler, J.A. (2007). Minichromosomes: The next generation technology for plant genetic engineering. Online Review. URL: www.isb.vt.edu.

Yu, W., Lamb, J.C., Han, F. and Birchler, J.A. (2006). Telomere-mediated chromosomal truncation in maize. *PNAS (USA)* 103(46): 17331-17336.

² See Section 2.2.1 of Current Awareness Report FW07007 for a description of Cre/lox recombination systems.

2.1.4. Mini-chromosomes in corn (MMCs) constructed *in vitro*

An alternative approach to engineering mini-chromosomes in corn via telomere truncations is to assemble an autonomous mini-chromosome (known as maize mini-chromosomes or MMCs) *in vitro* and then introduce this into a corn line. This approach has been recently described by Carlson and collaborating co-researchers from Chicago and North Carolina, USA.

The first mini-chromosomes employed in eukaryotic systems consisted of a simple centromere sequence from the yeast *Saccharomyces cerevisiae*, incorporated into a circular or linear yeast artificial chromosome (YAC) vector. Researchers used these yeast vectors to define a 125 base pair (bp) region of centromere sequence sufficient to support cell mitosis and meiosis. YAC vectors are able to incorporate very large (megabase) quantities of DNA and have been routinely used to manipulate large fragments of DNA for research purposes. Human artificial chromosomes (HACs) can also incorporate megabase quantities of DNA sequence and are used to introduce these DNA sequences into human cell lines. HAC formation has only been demonstrated in one particular human fibrosarcoma cell line, however, once established these HACs can be transferred to other mammalian cell types and are stably maintained. The establishment of *in vitro* constructed mini-chromosomes relies on the ability of the introduced centromere sequence to recruit the necessary DNA binding protein and other epigenetic (i.e. non-DNA) factors that enable it to be recognised and to function within the cell.

Corn centromeres contain repetitive DNA sequences similar to those found in mammalian centromeres. A series of deletion derivatives of B chromosomes from corn has been used to determine a 110 kilobase (kb) centromere sequence as the minimum size able to function in mitotic cell division. Carlson and co-workers used this knowledge of functional centromere sequence to develop autonomous mini-chromosomes in corn that do not necessitate the alteration of any endogenous chromosomes from the corn line. A library of corn genome sequences was searched for repetitive sequences, including those typically seen in corn centromeres. A site specific recombination system (Cre/lox)² was used to join selected sequences to a circular transformation vector containing an antibiotic resistance marker gene

(*nptII*) and a coloured cellular reporter gene (*DsRed*). A biolistic transformation system¹ was used to introduce these constructs into corn cells. Resultant transgenic plants were screened to determine if the transformation vector had integrated into the corn genome, or was carried as an autonomous mini-chromosome. An impressive 90% of the 52 transgenic events that were evaluated contained a mini-chromosome. While a number also contained an inserted construct, 83% contained only an autonomous mini-chromosome. This high rate of recovery of mini-chromosomes suggests that the tissue used in the transformation procedure (corn embryo tissue) is able to readily established mini-chromosomes if the correct centromeric sequences are provided.

Further analysis was carried out in detail on one of the mini-chromosome constructs (MMC1). When an analogous transformation vector that lacked the centromeric sequence was used to transform corn it resulted in the expected 100% insertion of the construct into the host genome. The construct that included the centromeric sequence gave 5/9 independent transformation events that resulted in an autonomous mini-chromosome. The remaining 4/9 events contained both an inserted construct and a mini-chromosome. Inheritance studies showed that the MMC mini-chromosome remained distinct from the host chromosomes and that the gene cassette it contained was stable through at least four generations. The genes carried on the MMC1 mini-chromosome were expressed and transmitted during cell division (mitosis) and chromosome partitioning during ovule/pollen cell development (meiosis). Notwithstanding this stability the MMC1 mini-chromosome was also able to be lost from the genome at a higher frequency than that of endogenous chromosomes.

The MMC1 mini-chromosome was shown to contain a 19 kb insert of corn DNA sequence. Nine kb was made up of 59-64 repeats of a previously identified centromere sequence (CentC sequence motif). The remaining sequence had homology to retrotransposon DNA sequences, also thought to be important in centromere function. This 19 kb insert is much smaller than a 69 kb rice centromeric region and the 110 kb corn B chromosome centromeric deletion region previously shown in other studies to be able to function in meiosis and mitosis. Data generated by Carlson *et al.* in this study supported this 19 kb sequence in MMC1 as able to function as a minimal requirement for inheritance of the mini-chromosome by meiosis or mitosis. However, due to the repetitive nature of the sequences involved the authors could

not rule out the possibility that the minimal region had expanded, contracted or re-arranged in some manner. The total size of MMC1 was small, at just 35 kb. However, much larger constructs, up to 200 kb, were also seen to form mini-chromosomes in corn that were able to be inherited, suggesting that the minimal MMC1 unit should be capable of accepting large donor sequences containing transgene cassettes and still function as a transmissible, autonomous mini-chromosome.

Source:

Carlson, S.R., Rudgers, G.W., Zieler, H., Mach, J.M., Luo, S., Grunden, E., Krol, C., Copenhaver, G.P. and Preuss, D. (2007). Meiotic transmission of an *in vitro*-assembled autonomous maize mini-chromosome. *PLoS Genet* 3 (10): 1965-1974.

2.1.5. Advantages of mini-chromosomes in plant biotechnology

Traditional methods to introduce ‘foreign’ genes into plants include *Agrobacterium*-mediated transformation and direct DNA transfer (e.g. biolistics). *Agrobacterium*-mediated transfer utilises the natural ability of the soil-borne bacterium *Agrobacterium* sp. to incorporate a region of its DNA into a plant genome. The DNA is transferred into the plant on a construct that has a size limitation and specific sequence element requirements. *Agrobacterium*-mediated transformation of plants is limited to those plants that (a) are susceptible to *Agrobacterium* infection, and (b) can be readily manipulated in an *in vitro* culture system. Direct DNA transfer methods introduce DNA sequence constructs directly into plant cells, with the hope that a portion of constructs can become incorporated into the host plant genome. Direct transfer methods can be used to introduce foreign DNA into plants that are not susceptible to *Agrobacterium* infection, however, they tend to be expensive and time consuming methods to use routinely.

Whilst these traditional methods of genetic engineering of plants have had many successes they do have their limitations. Both methods result in a random insertion event where the introduced gene construct can potentially be located anywhere in the host plant genome. Insertion may disrupt the host plant’s physiology by either disruption of endogenous gene expression, or by introduction of ‘non-anticipated’ gene products into the host plant from

chimaeric sequences generated at insertion junctions. These possible outcomes must be rigorously screened for before a transgenic line can be commercially developed and released. Random insertion of transgenes by traditional genetic engineering in plants also influences the predictability of gene expression and the level of gene product obtained.

The size limitations of the constructs able to be introduced into plants by these methods mean that only 1-2 gene expression cassettes are usually able to be introduced into a plant in a single step. In order to 'stack' genes, for example, to improve the spectrum of insect resistance, additional gene expression cassettes have to be introduced either via a secondary transformation of a primary transgenic line, or by crossing of transgenic lines. Both are time consuming options and both may result in gene expression cassettes located on different chromosomes within a host plant. These independent transgenic insertions will then segregate during subsequent breeding, such that only 1/16th of the progeny will retain the two stacked genes in a useful homozygous genetic state. Whilst this type of breeding system is used to stack genetic traits in commercial breeding of conventional crops it is time consuming and inefficient.

Another disadvantage of traditional transformation systems in plants is that of 'linkage drag'. This occurs when a portion of chromosomal DNA sequence adjacent to a trait of interest is non-randomly segregated in progeny. It occurs in traditional breeding programmes and can result in a difficulty in separating a deleterious trait from one cultivar from a desirable one in progeny of a cross. Many generations of backcrossing are often needed to introduce a good gene character into an elite cultivar without 'dragging' undesirable characteristics along as well. With traditional plant genetic engineering methods that randomly insert a transgene into the host plant genome, linkage drag can occur when a non-elite cultivar has to be used due to its amenability to transformation and the desired characteristic then has to be introduced into elite cultivars by traditional breeding.

The use of mini-chromosomes as platforms to introduce 'foreign' DNA into plants could overcome a number of these limitations. Gene expression cassettes introduced on mini-chromosomes would remain separate from the host plant genome. The introduced gene construct(s) would not influence the plant phenotype as issues such as disruption of native

genes by insertion events would not occur. Mini-chromosomes could act as platforms for the simultaneous introduction of multiple stacked gene traits, and/or could be added to or deleted from at a later stage to readily develop new transgenic lines. As well as enabling wider spectrum insect resistance, gene stacking allows for increased production of a transgene product and would facilitate the commercial production of compounds of pharmacological value in transgenic crops. It is conceivable that an entire metabolic pathway could be introduced into a plant system on a mini-chromosome platform. The introduced transgene(s) would be retained as a discrete genetic unit (independent linkage group) that would be inherited in a predictable manner without linkage drag and the need for long breeding programmes to integrate transgenes into desirable cultivars. The predictability of inheritance of the mini-chromosome unit would also allow traditional breeding methods to be used for the ready removal of the entire transgene complement of a line if required.

2.1.6. Implications of mini-chromosome gene transfer systems on GMO testing and regulation

Mini-chromosome gene transfer systems are unlikely to have any major impact on testing for GM events in foods. The genetic makeup of the mini-chromosome construct would be known to the developer in the same manner as it is currently known for insertion/integration transgene events. So long as the developer is required to provide this information to regulators, molecular tests to determine the presence of transgenes should be able to be established and utilised in the same way as for current GMOs. The only issue may be the logistics of detecting each of a large numbers of transgenes in multiple trait, stacked gene events if a precise identification of an event is required.

Regulation of GMOs developed using mini-chromosome platforms would be the same as current integration/insertion events. The only major issue that might arise is when endogenous sequences only are utilised in the construction of a mini-chromosome, giving the argument that no 'foreign' DNA is present in the organism. This is the same argument as is being posed for the use of cisgenic and intragenic transformation vectors³ and is not specific to the use of mini-chromosomes.

³ See Section 2.2 and 2.3 of Current Awareness Report FW0771 for a discussion of cis- and intragenic transformation vectors

2.2. Use of RNA Interference techniques (RNAi) in biotechnology

While agronomic performance and crop yield remain important key targets in engineering food crops there is an increasing emphasis on development of GM plants with ‘2nd generation’ output traits. These are traits that benefit the processor and/or consumer. One area of output traits of particular interest is the improvement of nutritional value in targeted crop plants.

While traditional breeding practices have been very successful at improving the nutritional value of food crops, the process is time consuming and limited by genetic material available for breeding programmes. This latter limitation includes natural species barriers to breeding as well as loss of gene pools due to domestication and breeding of crop plants. Another major barrier to natural breeding for improved nutritional value is that beneficial traits in food organs of plants, such as seeds or fruits, may be deleterious to other tissues of the plants such as vegetative tissues, and issues of ‘linkage drag’ (see Section 2.1.5) can make it difficult to introduce a particular positive trait without associated negative ones. Recent advances in the genetic engineering of plants have opened up extensive possibilities for improving the nutritional value of food crops. Ongoing research in gene mapping and identification of genome sequences for a range of model and crop plants provides information to enable the directed manipulation of metabolic pathways in plants. Similarly the –omics disciplines of transcriptomics, proteomics and metabolomics are proving to be important in understanding plant metabolic pathways and how they could be manipulated.

To date the GM crops that predominate in the food market are the so called ‘1st generation’ GM plants. These plants have been engineered with novel ‘input’ traits that benefit the grower, such as herbicide and pest resistance. Engineering of the crop requires the insertion of an expression cassette for a single novel transgene into the plant (for example the *cry* genes in Bt-crops). Manipulation of metabolic pathways for the modification of crop nutritional value is more complex than this single gene insertion event. Metabolite engineering often requires turning off an endogenous gene or reducing the level of expression of an endogenous gene. Due to the complexity of biosynthetic pathways and the presence of

feedback metabolic loops to regulate levels of metabolites in a plant it is often necessary to suppress, introduce or regulate the expression of several genes together to obtain the desired metabolic effect. A secondary issue with engineering metabolic pathways is that they are often involved in a number of physiological systems within a plant and targeted engineering of expression is required in order not to disrupt the entire plant system. Engineering of metabolic pathways therefore requires a high degree of control over site and level of gene expression.

Recent engineering of metabolic pathways in plants has utilized the natural plant regulatory mechanism of RNA interference as a means of regulating levels of gene expression. During the reporting period researcher Tang *et al.* (2007) reviewed current RNA interference methodologies and some of the application they have recently been used for in engineering crop plants. An introduction to RNA interference methods is provided along with a summary of information in the review.

2.2.1. Background to RNA interference

The central dogma of genetics is that genes are expressed in cells via a messenger molecule (messenger RNA) that directs protein synthesis in a specific manner (see Fig 5). While DNA is a double stranded molecule RNA is single stranded, and mRNA has a sequence that is complimentary to the coding (sense) strand of the DNA genetic blueprint.



Figure 5 Biological information flow

Taken from: www.en.citizendium.org

RNA interference (RNAi) is a process that disrupts the flow of genetic information from DNA to protein synthesis. The process occurs naturally in many organisms including plants. The distinguishing characteristic of RNAi is the destruction of mRNA molecules by double

stranded RNA (dsRNA) 'trigger' molecules. RNAi has an important biological role in gene regulation and in the protection of organisms from genetic parasites such as dsRNA viruses.

The discovery of RNAi was a major breakthrough in biological research. It was the unexpected outcome of attempts to genetically engineer the colour of flower petals in the early 1990s. Researchers in the Netherlands were attempting to enhance the colour of petunia petals by introducing extra copies of a gene involved in pigment biosynthesis. While some plants did show enhanced petal colour others showed petal sectors with lost pigmentation. Molecular characterisation of the white tissue showed that mRNA from both the endogenous gene and the introduced 'extra' gene copies for pigment biosynthesis were absent. This phenomenon was given the name 'co-suppression of gene expression', however, the mechanism was unknown. It was also seen that the introduction of a transgene into a plant that resulted in the expression of the non-coding strand of a DNA molecule as mRNA (called antisense RNA) could result in suppression of endogenous gene expression. In the late 1990s researchers Andrew Fire and Craig Mello, working with a model nematode, found that traces of dsRNA in the organism resulted in dramatic silencing of genes that contained sequences identical to those in the dsRNA. They called this 'RNA interference' and in 2006 were awarded the Nobel Prize in Physiology or Medicine for their research into this phenomenon. At the same time plant researchers found that simultaneous expression of sense and antisense transgenes in a plant resulted in more efficient silencing of both endogenous and introduced genes. A detailed examination of this process revealed that both co-suppression and sense/antisense gene silencing resulted from the formation of dsRNA molecules in the cell, resulting in RNAi.

There are a variety of RNAi pathways, with different outcomes in eukaryotic cells, however, they all involve the same basic steps (see Fig 6).

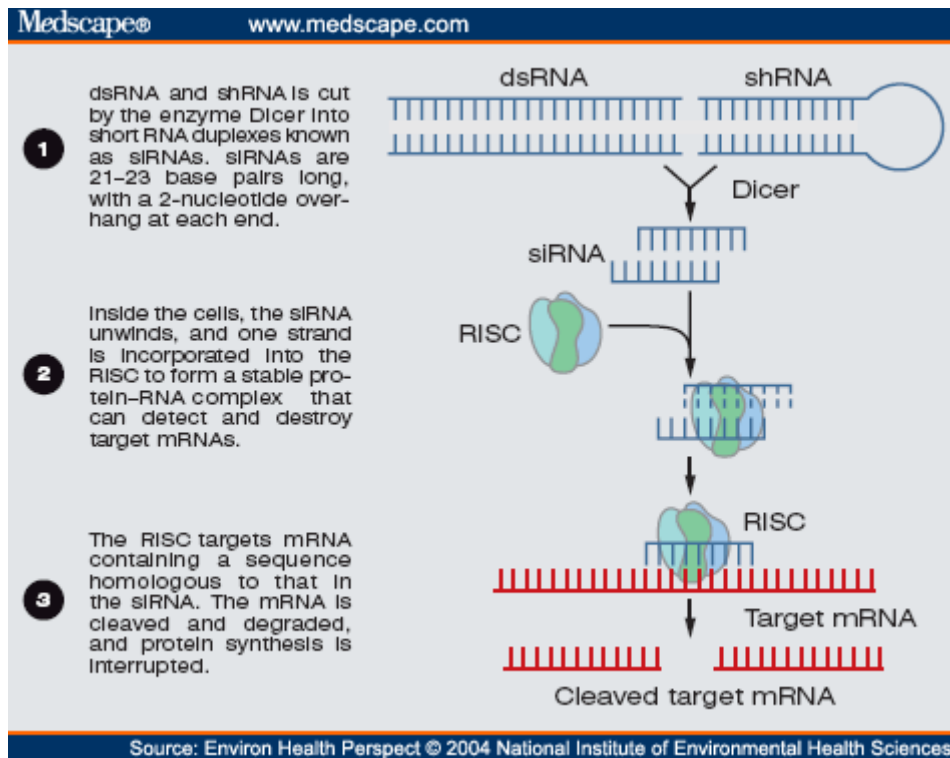


Figure 6..Basic steps in RNAi gene silencing

1. Initiation of pathway by (i) large dsRNA ‘trigger’ molecules, or by (ii) microRNA molecules (shRNA) that contain a degree of imperfect match which enables double stranded base pairing via a hair-pin loop structure.
2. Cleavage of ‘trigger’ or microRNA molecules by specialized RNA cleaving enzyme called ‘Dicer’ into small dsRNA molecules (si- or miRNA). These molecules are 20–25 nucleotides in length with a two bp overhang at each end.
3. Association of si/miRNA molecules with proteins to form an active RNA induced silencing complex (RISC). These active silencing complexes then target and cleave mRNA molecules with complimentary sequence to the small dsRNA sequence in the complex.

The outcome of RNAi in a plant can be complete suppression of gene expression or a down–regulation of gene expression. RNAi-mediated gene silencing can spread through plants from one region to another, possibly thorough pores that connect cells together.

2.2.2. RNAi methods used in biotechnology

While the first demonstration of dsRNA mediated gene silencing in plants was via crossing parents that over-expressed sense and antisense RNA, subsequent methods have employed specific transformation vectors designed to deliver dsRNA molecules into the plant. Initial RNAi vectors were designed to generate long dsRNA molecules with the same sequence as the target gene. Vectors were then also developed to express hairpin RNA molecules with complementary sequence to the gene of interest. It was observed, however, that constitutive expression of ds or hairpin RNA of some genes in plants often led to unexpected and undesirable effects on plant growth and development. Chemically inducible RNAi silencing vectors were developed to enable spatial and temporal control of gene silencing. Whilst often effective, these chemically-induced vector systems are generally not appropriate for large commercial crop systems.

Long dsRNA molecules produced using RNAi vectors have been shown to activate the phosphokinase response (PKR) pathway in mammalian cells; a pathway involved in cell response to external stress signals. Plant cells also express PKR pathway genes and may have a similar stress response pathway. Avoiding potential activation of this pathway is important in order to avoid unintended effects of dsRNA gene silencing. In contrast to dsRNA, endogenous expression of microRNA molecules in plants and animals has not been seen to be associated with adverse effects other than the target gene regulation. Second-generation RNAi vectors are now being designed to produce the short miRNA molecules that result from 'Dicer' cleavage of longer microRNA molecules. Plant artificial miRNAs have been developed and used successfully to silence a variety of plant endogenous genes and genes of plant pathogens. A major advantage of miRNA mediated RNAi gene silencing is that miRNA expression is subject to temporal and spatial regulation. The ongoing dissection of miRNA structure should provide extra options for tissue specific RNAi gene silencing.

2.2.3. Examples of RNAi in plant biotechnology

Increasing the content of lysine in specific crop plants (e.g. corn) continues to be a target for genetic engineering. Lysine synthesis is strongly regulated by a feedback inhibition loop in which lysine itself inhibits the activity of the first enzyme (DHPS) in the biosynthetic

pathway to lysine production. Natural genetic mutations in the tobacco gene for this enzyme make it insensitive to lysine feed back inhibition. This results in over production of lysine in all plant tissues. However, while beneficial in seed, these increased lysine levels can cause abnormal vegetative growth and flower development, which in turn reduces seed production. Similar bacterial DHPS genes have been isolated that are insensitive to lysine feed back inhibition and these have successfully been introduced into plants using a traditional transgene cassette which targets expression of the novel introduced gene in seed (LY038 high lysine corn is an example of this methodology). However, seeds of plants that accumulate high levels of lysine show poor germination as the high levels of lysine in the seed are not efficiently degraded as the seed germinates. An RNAi mediated gene silencing approach has been used to overcome these difficulties in generating high-lysine corn plants. Corn contains a group of 22-kD zein storage proteins that are abundant in the seeds, but which are low in lysine content. This makes corn a nutritionally poor food if eaten as a staple diet. Down regulation of expression of the 22-kD zein gene by RNAi has been used to generate normal corn seed with high levels of lysine-rich proteins as well as free lysine.

Another nutritionally important crop is cotton. While cotton is mainly used for fibre production there is a large amount of cotton seed left as a bi-product of processing. These cotton seeds could potentially be used as a source of dietary protein and calories by large numbers of people in developing countries, except that cotton seed contains a toxic gossypol terpenoid compound. As the gossypol terpenoid is important to the cotton plant as protection from insects and other pathogens any method used to reduce its content in cotton for nutritional reasons should be limited to the seeds only. Transgenic cotton plants have been produced that express an RNAi construct of a gene in the gossypol biosynthetic pathway, fused to a seed-specific promoter. Reduction of gossypol levels were seen in seed, while the content in non-seed tissues of the transgenic plant was comparable to control non-transgenic plants.

While insufficient food calories is a major issue in developing countries, an excess of digested calories, leading to obesity and other diseases is of increasing concern in developed nations. Foods that are rich in inefficiently digested carbohydrates, such as fibre, are increasingly looked to as health promoting. The major source of plant carbohydrate is starch.

Starch is made up to two molecules, amylose and amylopectin. In cooked foods that undergo cooling before eating, amylose molecules tend to form digestion-resistant complexes. These complexes are part of healthy natural dietary fibre. With the aim of increasing the amylose content of wheat grains, an RNAi construct was designed to silence the genes for two of the enzymes in the pathway that converts amylose to the amylopectin molecule. This construct was under the control of a seed specific promoter, and resulted in increased amylose content in the grains of the transgenic wheat plant.

Before its molecular mechanism had been elucidated RNAi technology was used in the development of the Flavr Savr tomato. To delay ripening during shipment an antisense RNA construct was introduced into tomatoes to reduce the level of synthesis of the plant ripening hormone ethylene. More recently RNAi gene silencing has been used to improve the carotenoid and flavonoid levels in tomato fruits. These compounds are thought to have strong antioxidant activities and to be beneficial to health. Biosynthesis of these compounds in tomato is regulated by a gene that is also involved in several other light-regulated pathways in tomato plants. Traditional approaches to silencing this regulatory gene in tomato have shown detrimental effects on several phenotypic characteristics including abnormal plant growth. An RNAi approach has been used to successfully suppress expression of this regulatory gene, specifically in tomato fruit. The fruit of transgenic plants developed using this method showed increased levels of carotenoid and flavonoid compounds, with minimal effects on plant growth and other fruit quality parameters.

These are just some examples of the successful use of RNAi technology to improve nutritional value of food plants. As more target genes involved in metabolic pathways are discovered using –omic technologies and biosynthetic pathways in plants are unravelled the opportunities to modify these pathways using RNAi gene regulation methods are likely to increase dramatically in the near future.

Sources:

Tang, G., Galili, G. and Zhuang, X. (2007). RNAi and microRNA: breakthrough technologies for the improvement of plant nutritional value and metabolic engineering. *Metabolomics* 3: 357-369.

Background:

RNA interference. Online article. URL: http://en.citizendium.org/wiki/RNA_interference

2.2.4. Implications of RNAi technologies for testing and regulation of GM foods

The use of RNAi technology for the generation of transgenic plants has some implications for both testing and regulation of GM foods. Whilst the methodology involves the introduction of an expression cassette into the plant in the same way as more traditional gene insertion engineering, with RNAi the gene sequence involved is often complementary to endogenous gene sequences already found in the plant genome. This makes design of tests to detect inserted genes more difficult. Such a test would rely on the presence of additional elements in the expression cassettes such as tissue specific promoters and a precise knowledge of the DNA sequences involved. Availability of generic tests to screen for transgenic plants would be less likely and more reliance would need to be placed on specific tests for each particular transformation event. As with current event specific testing this would rely on provision of information on specific gene sequences being a requirement of regulatory procedures.

With respect to general regulation of foods derived from plants engineered using RNAi technology:

- The use of RNAi techniques to effectively manipulate plant metabolic pathways and generate food crops with altered nutritional properties will require the ongoing, case-by-case assessment of safety of these crops as foods⁴. Ongoing debate over the safety of high lysine corn illustrates the public concern over safety of metabolite engineering.
- The argument has already been put forward that plants engineered by RNAi to suppress endogenous gene expression fall into the category of cis-genics and as such

⁴ See Section 3.1 of Current Awareness report FW0771 for a discussion of safety assessment of 2nd generation transgenic foods.

should be exempt from current regulations⁵. As more emphasis is placed on the generation of GM crop plants with engineered metabolic pathways, and use of RNAi techniques increases, these debates on regulation of GM foods are likely to continue and to require resolution.

3. FOOD SAFETY AND COMPOSITION STUDIES

3.1. Novel technique to profile amino acids in GM foods for the assessment of equivalence

In the safety assessment of GM plants as food the aim is to determine whether the GM food is as safe and as wholesome as food from a conventional, non-GM equivalent of the plant (either the parent line from which the GM plant was derived or a near-isogenic line⁶). The demonstration of substantial equivalence is central to this assessment. 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. Current safety assessments target equivalence between the GM food and its non-GM comparator in terms of 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.

Currently unintended changes are assessed in a somewhat limited manner by assessment for targeted factors (eg: specific nutrient, antinutrient and toxicant composition). It has been suggested that a variety of other cellular constituents could be measured to further inform equivalence assessments, particularly in the determination of unintended changes to the plants composition. These include comparative profiles of gene transcripts (transcriptomics), total protein profiles (proteomics) and profiles of metabolites (metabolomics). A number of studies have been undertaken to look at these types of analyses for GM food safety

⁵ See Section 2.2 of Current Awareness Report FW0771 for a discussion of cis-genics and regulatory issues.

⁶ Definition of isogenic: Genetically identical (except for sex). Coming from the same individual or from the same inbred strain. www.biochem.northwestern.edu.

assessment. While potentially useful, these assays are currently considered to be not well enough developed and to lack sufficient control data on degrees of natural variation in compositional levels to provide the rigor and validation required for safety assessment of foods⁷.

During this reporting period a group of researcher from Spain (Herrero *et al.*, 2007) published a method to determine and compare chiral amino acids in GM and conventional corn and suggest that this method may provide useful additional data to help support safety assessment of GM foods. An introduction to amino acids and chirality, and why this should be targeted in an assay is provided, along with a summary of the published work.

3.1.1. Introduction to amino acids and chirality

Amino acids are biochemicals that make up the building blocks of proteins and also function as intermediates in cellular metabolism. They have a basic structure that includes a nitrogen-containing amine group at one end and a carboxyl group at the other end (see Fig 7). The ‘R’ group of the molecule is variable and this allows for the range of amino acids present in cells.

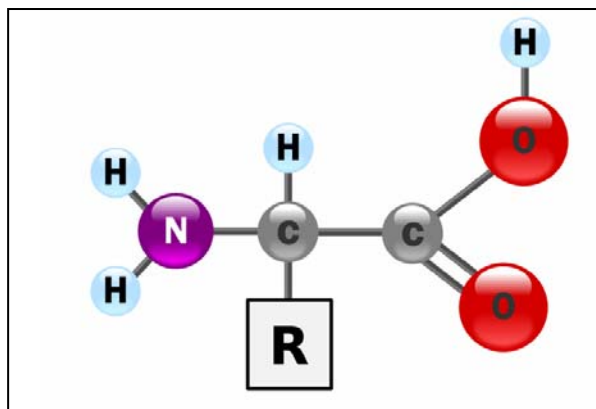


Figure 7 Basic structure of an amino acid

Taken from: www.wikipedia.org

A group of twenty amino acids is utilized by cells to synthesize proteins. The amino acids are joined together like beads on a string and the string can then fold and twist around itself (see Fig 8). The specific amino acid content of the string and the relative position of the amino acids are determined by the DNA sequence of the gene encoding that protein. The

⁷ See Section 2.2 Current Awareness Report FW07007 and Section 3.1.1 Current Awareness Report FW0771 for discussion of –omics methodologies in safety assessment of GM foods.

molecular structure and chemical properties of the various amino acids along the string determines the 3-D shape of a protein, influencing its stability and ultimately the protein's functionality. Proteins are essential to cellular metabolism and provide a range of functions. These include enzymes to facilitate metabolic reactions, structural molecules, storage molecules, transport molecules, antibodies involved in cellular defence mechanisms, and hormones.

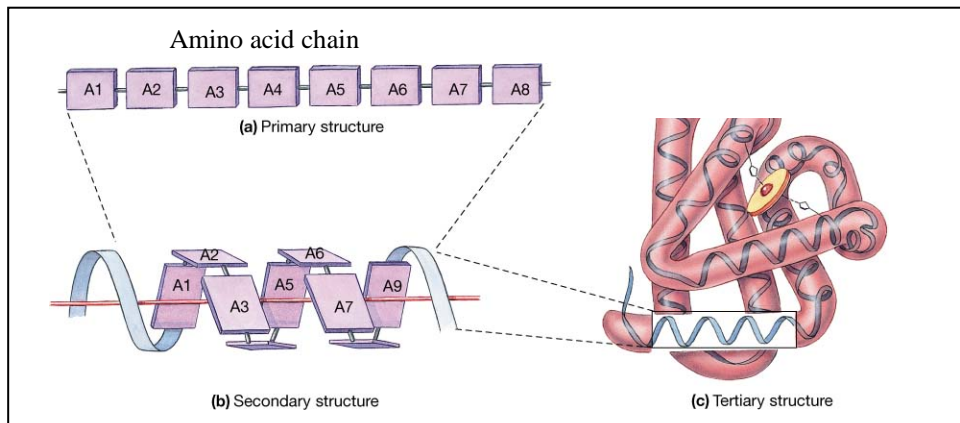


Figure 8 Amino acids joined to give a protein molecule

Taken from: www.stat.rice.edu

Amino acids can also function in the cell as intermediates in cellular metabolism. The twenty amino acids involved in protein synthesis can be used to synthesize other biomolecules or can be converted to urea and carbon dioxide as a source of energy for the cell. A number of non-protein amino acids are also found in cells and can have a range of functions. For example, the cyclic non-protein amino acid aminocyclopropane-1-carboxylic acid is involved in the production of the plant hormone ethylene. In humans the thyroid hormones are non-protein amino acids.

The basic amino acid structure is asymmetrical about the central carbon atom and can therefore exist as mirror image molecules. That is, the groups attached to the central carbon atom can have different spatial orientations. A chiral molecule is one that is not able to be superimposed on its mirror image; like left and right hands are made up of the same components in the same order but are not able to be superimposed. With the exception of glycine, where the R- group is a hydrogen atom, amino acids exist as chiral molecules (see

Fig 9). While amino acids can be found in both left and right handed forms (L- and D-forms), all amino acids found in proteins exist naturally in the L-configuration. D-amino acids are therefore not naturally found in proteins and are not involved in metabolic processes in eukaryotic organisms such as plants and humans. Free amino acids in the D-form have been shown to comprise only about 0.2-8% of the free amino acid content of plants. D-amino acids have been shown to potentially have an involvement in aging and disease in humans.

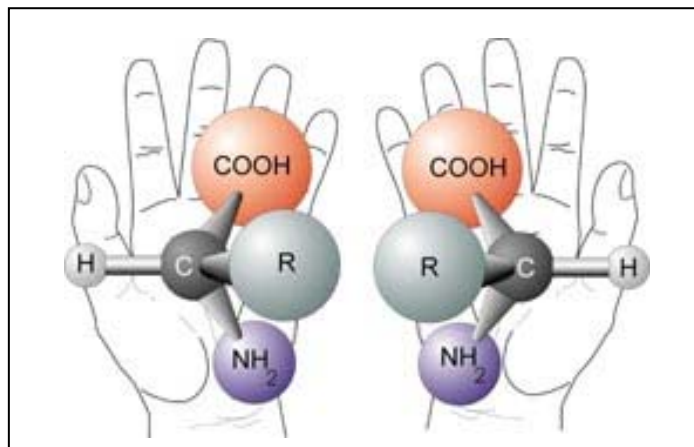


Figure 9 Chirality of amino acid molecules

Taken from: www.web99.arc.nasa.gov

The chiral configuration of an amino acid can influence the biological structure of a protein. L-amino acids naturally join to form a chain that twists in a right-handed alpha-helix. Experimental joining of D-amino acids into a chain forms a left-handed alpha-helix. The right-handed twist has been shown to be more stable than the left-handed configuration. Hence an alteration in the proportion of L- and D-amino acids in an organism could have significant effects on protein synthesis, leading to faulty proteins. Due to the close identity of D- and L- forms of amino acids the addition of one form to a metabolic system where it is usually absent could cause it to act as a competitive inhibitor in the system.

3.1.2. Significance of amino acid chirality in foods from GM plants

The significance of chirality of amino acids in foods from GM plants can be considered at two levels:

1. The chirality of the amino acids present in the GM compared to the non-GM plant and the impact of any alterations to the proportions of L- and D- forms on the metabolism of the GM plant. Such alterations could result in unintended metabolic effects in the GM plant that may be difficult to detect with current targeted compositional testing.
2. The implications of chirality for amino acids in the diet. Amino acids taken in the diet can have one of two fates. Either they are incorporated into proteins or they are broken down for energy and metabolic intermediates. They are not stored and the body does not excrete them intact, therefore they must be metabolized in some way. The utilization of D-amino acids in the diet varies depending on the amino acid. Free D-phenylalanine can act as a nutritional source of L-phenylalanine, while D-lysine is devoid of any nutritional value. High levels of D-tyrosine in the diet have been shown to impair growth of mice. Any alterations to the proportions of L- and D- forms of amino acids in a GM plant could therefore have implications for nutritional adequacy of amino acids in the diet with respect to their ability to function in normal metabolic processes.

Herrero and co-workers from the Institute of Industrial Fermentations in Spain have proposed that a characterization of the chirality of amino acids in GM plants could therefore add valuable data to inform the substantial equivalence assessment of GM food safety.

3.1.3. Analysis of chiral amino acids in conventional and transgenic corn

Herrero *et al.* (2007) developed a new, rapid method to identify and quantify L- and D-amino acids. They then used this method to investigate three lines of transgenic corn, and their corresponding non-transgenic parental lines, grown under identical conditions.

Standard analytical methods such as High Pressure Liquid Chromatography (HPLC) and Gas Chromatography (GC) can be used to analyze chirality of amino acids, however, they have several drawbacks for the analysis of GM food safety. They require expensive materials, have lengthy sample preparation requirements and a relatively lengthy analysis time (usually ~ 50 mins). They are also unable to detect some amino acids without complicated

derivatization steps. For example, the derivatization required to detect amino acids prior to GC can involve more than 10 steps. This prompted the researchers to develop a new analytical method to detect chiral amino acids which utilizes capillary electrophoresis (CE). The complete method involves the combination of micellar electrokinetic chromatography (MEKC) with a chiral selector and laser-induced fluorescence (LIF). The methodology, known as chiral-MEKC-LIF, is described in detail in the publication. It is considered to be fast and reproducible, and allows quantification with good efficiency and sensitivity. Using algal samples the complete separation of a range of L- and D- amino acids (D- and L- forms of arginine, leucine, asparagine, serine, alanine, glutamine and aspartate) was achieved in less than 25 minutes. The authors recognised some limitations in the ability to separate all amino acids using the method but feel further optimization of the method can overcome this.

This methodology was then applied to three commercial transgenic corn lines and their non-GM parental lines, grown in identical field conditions in Spain. All of the lines contained an introduced insecticidal *cry* gene. The corn lines were Aristis (wild type and its Bt transgenic variety), Tietar (wild type and its Bt transgenic variety) and PR33P66 (wild type and its Bt transgenic variety). The transgenic and non-transgenic nature of the corn samples was confirmed using molecular characterisation. The identification and quantification of ten free L/D amino acids was then performed. The L- and D- forms of arginine, alanine, serine, glutamine and asparagine were all identified in the investigated corn varieties. Results showed that the method could detect as low as 1% D-arginine in a background of more than 99% L-arginine. The different varieties of parental corn showed different profiles of L- and D-amino acids. The three varieties were statistically different in their content of all but D-glutamine. This result is consistent with expected natural variation in composition of conventional corn lines.

The L- and D-amino acid profiles of the parental lines were then compared to their GM varieties:

- For Tietar/Tietar-Bt the % D-values were equivalent for all amino acids identified. This would suggest that in this GM line the insertion of the *cry* gene did not modify any metabolic pathway linked to the detected amino acids.

- For Aristis/Aristis-Bt significant differences (less than 5% likely to be due to chance) were seen in the % D- content of the amino acids arginine, serine and asparagine.
- For PR33P66/PR33P66-Bt significant differences (less than 5% likely to be due to chance) were seen in the % D- content of arginine, serine and alanine.

No comment was made on whether the statistically different levels of D-amino acids seen in the latter two transgenic lines fell within the natural variation seen between all of the conventional parental lines. The authors were unable to provide any physiological explanation, based on known *cry* gene effects on metabolites in corn, for the differences seen in L/D- amino acid profiles for the Aristis and PR33P66 corn parental/transgenic comparisons.

Overall these results support the use of chiral amino acid analysis to provide additional data to enhance determination of the substantial equivalence of a GM plant and its non-GM counterpart. Changes in the L- and D-amino acid ratio in a transgenic plant could indicate unintended modifications to the plant's metabolism related to the insertion of the transgene. The health significance of such changes is not yet confirmed, however, the information able to be provided by the method described in this publication could open up new perspectives in the study of chemical composition and safety of food from both conventional and GM plants.

Source:

Herrero, M., Ibáñez, E., Martín-Álvarez, P.J. and Cifuentes, A. (2007). Analysis of chiral amino acids in conventional and transgenic maize. *Anal. Chem* 79 (13): 5071-5077.

3.2. 90-day rat feeding studies revisited

3.2.1. Background to the use of animal feeding studies in food safety assessment

The need to develop approaches to assess the safety of whole foods was first highlighted in the 1950s when food irradiation was introduced, followed by development of novel proteins from fungi in the 1960s. The first guidelines for safety assessment of whole foods were set up in 1969 and 1970 by the “Protein Advisory Group for Single Cell Protein”, and in 1984 by the UK Department of Health and Social Security. Use of gene technology for the generation of novel foods first appeared in the 1980s and the challenge for assessing the safety of foods derived using this technology was considered by a number of national and international regulatory bodies (e.g. FAO/WHO in 1991).

In 1997 the EC issued its regulation concerning novel foods and food ingredients, including GM foods (EU Regulation 258/97). Recommendations contained in these regulations, and based on the opinion of the Scientific Committee on Food (SCF), suggested a case-by-case approach where chronic or sub-chronic animal feeding studies may be appropriate when preceding data does not enable an acceptable settlement of safety issues. The SCF had expressed its doubts about the usefulness of animal feeding studies, both from a scientific and from a safety point of view. Issues they raised included:

- Difficulties may be encountered in the interpretation of data from animal feeding trials when high levels of the novel food are included in the animal’s diet. This may be due to general distortions in the nutritional profile of the diet unrelated to the genetic modification.
- Animal feeding studies do not allow for the use of large uncertainty factors that are generally applied for safety assessment of food additives.

The SCF suggested a decision–tree approach to safety assessment of novel foods, including an optional requirement for animal feeding studies to provide missing information. However, as the SCF gave no specific advice on how to do these feeding studies, the design of meaningful animal feeding trials for safety assessment of whole foods has long been a matter of dispute. An illustration of this is the recent debate around the methodology used by

Monsanto Inc in the feeding trial they carried out for safety assessment of their GM corn line MON863.

In 1999 the EC funded a series of research projects under an umbrella title of ENTRASFOOD. The aim of the projects was to develop and validate the scientific methods needed to enable the assessment of GM foods in accordance with the EC regulations of 1997. One of the projects was “New methods for safety testing of transgenic food” (SAFOTEST). The results of a 90-day feeding study design developed in this project have just been published as a number of referred publications and were reviewed by Knudsen and Poulsen (2007). The focus of this feeding trial design was to distinguish between primary effects related to the presence of the transgene product and any other secondary effects caused by feeding the GM food. The overall aim of a safety assessment for whole foods derived from gene technology is to determine whether the food has the same level of safety as its traditional (non GM) counterpart, based on a hazard assessment and an exposure (intake) assessment. Hazard assessment can include new toxicological, allergenic and nutritional aspects of the genetic modification. The SAFOTEST project focussed on hazard assessment for new toxicological and nutritional issues but not on allergenicity assessment as this requires a different approach⁸

3.2.2. Design of a rat 90-day feeding trial to distinguish between primary and secondary effects of a GM event

The most common approach previously used to test the safety of GM plant food in a rodent feeding trail is to formulate separate commercial diets containing the GM food and its conventional counterpart (near isogenic line) at levels of 11% and 33%. As in the study of MON863 corn presented by Monsanto, this allows for the testing of levels of 11%, 22% and 33% of the GM material in a commercial diet. This approach does not rely on any background knowledge of other issues addressed in the safety assessment of the GM food. While this enables a direct biological comparison between the GM and the control diet, it does not allow for distinction between effects due directly to the inserted GM gene product and effects due to other issues with the GM food line.

⁸ See Section 2.2.2 of Current Awareness Report FW07007 for information related to the assessment of allergenicity of novel proteins in GM foods.

The feeding trial design developed in the SAFOTEST project relies on information obtained in other areas of the safety assessment of the GM food and is dependant on this information being generated and utilised in the correct order:

1. Produce and characterise the transgenic line and parental (comparator) line of the food plant.
2. Produce the novel gene product(s) in a pure form, e.g. production and purification of a recombinant protein from a bacterial system.
3. Perform a detailed compositional analysis of the food products from the transgenic and parental food lines.
4. Perform a 28-day rodent study to test the toxicity of the novel gene product(s) expressed in the transgenic line in order to determine their *Lowest-Observed-Adverse-Effect-Level* (LOAEL) based on the use of sensitive and specific biomarkers.
5. Perform *in vitro/ex vivo* toxicity screening tests of the novel gene product(s) to determine specificity and sensitivity of biomarker assays. i.e. establishment of which organ(s) and associated biomarker assay(s) are affected by LOAEL doses within the target organism.
6. Design a purified diet taking into account maximum tolerance of the GM food material in the diet and the total and relative content of macro-and micro-nutrients in the parental and GM food items.

In the SAFOTEST project three GM rice lines were developed for use in the design of the rat feeding trial. Two lines expressed a novel plant lectin gene (one from kidney bean and the other from snowdrop), while the third line expressed an insecticidal Bt toxin gene from *Bacillus thuringiensis*. It was determined that rats could tolerate up to 60% of their diet as ground rice seed meal. To use this level in the feeding trials necessitated the generation of a large amount of GM rice and so the GM lines and near isogenic comparator lines were grown in bulk in field plots in China. A detailed comparison was carried out between the GM rice lines and their comparators for chemical composition as well as for pesticide residues and microbial quality. This enabled the development of a purified diet that accounted for any significant differences in composition between the GM lines and the comparators. Based on

prior information from rat feeding studies differences were adjusted for where the component made up 5% or greater of the food material.

A 28-day toxicity trial was used to determine the LOAEL for the transgenic rice lines. The researchers intended to then use advance molecular methodologies (including microarray) on *in vitro/ex vivo* tissue samples to determine which organs and associated biomarker assays should be targeted in the analysis of the 90-day feeding trial. Unfortunately it was found that the types of molecular assays used in these studies require further development and standardisation before they can give reliable results for this type of system. In this case the 28-day feeding study and an acute oral toxicity study were able to provide information on which biomarker assays should be used to determine toxicity in the 90-day feeding trial. The lesson from the SAFOTEST project results reinforced that identification of relevant target organs and their associated biomarkers should be informed by the 28-day feeding trial and then confirmed using *in vitro/ex vivo* studies, and that this should be done before finalisation of the 90-day feeding trial parameters.

The 90-day feeding trial was based on the OECD Repeated Dose Toxicity Study guidelines No.408, with respect to parameters such as number of animals per group, duration of study and overall clinical, biochemical and pathological examinations to be performed. The main differences from the OECD guideline were:

- Feeding a whole food (in this case rice seed meal) mixed in the animal diet, rather than just a chemical.
- The number of treatment groups and the type of treatment.

The SAFOTEST feeding trial design contained three treatment groups:

1. Control – parental rice at a level of 60% in a basic purified diet.
2. Test 1 – GM rice (containing expressed transgene product) at a level of 60% in a basic purified diet.
3. Test 2 – GM rice (containing expressed transgene product) at a level of 60% in a basic purified diet GM rice and spiked with purified transgene product at LOAEL.

The spiking procedure in Test regime 2 was designed to distinguish between gene product related effects of the GM food and any other secondary effects. The expectation was that

after 90 days the individuals given the spiked diet at LOAEL would show the same type and level of adverse effects as seen in the previous 28-day trial when purified gene product was fed at LOAEL. If the same type of adverse effects seen in the 28-day trial were to be seen in the 90-day trial in individuals fed the GM food only (unspiked) it would suggest that the level of transgene product expressed in the food was sufficient to cause, and was responsible for the adverse effects. If any different adverse effects from those seen in the 28-day trial using purified gene product were seen in either treatment group fed GM food in the 90-day trial, compared to the non-GM counterpart, this would suggest a secondary issue with the GM food, unrelated to the gene product *per se*. Either of these results would then require further safety evaluation of the GM food. Alternatively, if no adverse effects of any type were seen in the test group fed the GM food only, then the food can be considered safe by comparison to the non-GM counterpart in a 90-day rodent feeding trial.

In the review of the SAFOTEST project the results of a 90-day feeding trial for one of the rice lines containing a lectin gene (from kidney beans) were presented. At 60% GM feed in the diet the amount of expressed gene product in the feed was 30mg/kg body weight/day. This equates to 100-200 times greater than the human consumption intake of rice in Europe and so reflects a high margin of safety with respect to extrapolation of results to human safety. In the spiked diet there was an additional 70mg/kg body weight/day (LOAEL) of purified lectin. Using the feeding treatment groups outlined above for 90-days, and testing for a range of biomarkers (e.g. plasma sodium, protein, urea, weight of stomach, etc), the researchers were able to determine that the GM food alone contained high enough levels of the lectin gene product to cause a number of similar effects to those seen with the spiked diet. This demonstrated the ability of this feeding trial design to determine specificity of effects of the GM food on rodents in the trial.

The strength of this feeding trial design is the ability of the spiking procedure to pick up effects relevant to the GM food being tested. The spiking regime should be designed on a case-by-case basis depending on the GM food involved and its intended effects. If the purpose of the GM food is unrelated to nutritional composition, or is intended to specifically reduce the level of a toxicant in the food then the best approach is to spike the diet of the group fed the GM food. This will enable the demonstration of equivalence of the GM food in

a diet or, if appropriate, of reduction or removal of any toxicant in the GM food, and will indicate any secondary issues associated with the GM food not related to the transgene product. If, on the other hand, the purpose is to increase the presence of a nutritional compound in the GM food, the diet of the control group fed the non-GM comparator would need to be spiked in order to show the positive effect of the GM product in the diet.

The review emphasises that the SAFOTEST approach should not be seen as a 90-day rodent feeding trial only, but as a more comprehensive comparative safety study, designed to use biological screening of health effects to determine the relative safety of a GM food compared to its non-GM counterpart. The feeding trial design is able to separate unintended safety aspects of the novel gene product introduced into the GM food from unintended safety issues associated with the GM plant but unrelated to the novel gene product. These secondary safety issues could result from the regeneration of the GM plant after introduction of the transgene. The complete data set that is derived from the SAFOTEST approach includes information on the parent plant(s), the gene construct(s), the gene product(s), data from compositional analyses, data on toxicity of the purified transgene product from 28-day rodent toxicity trials, and from *in vivo/ex vitro* toxicity studies as well as toxicity data for the complete GM food from the 90-day rodent feeding trial. Much of this data is already required in safety assessments for GM foods under current regulations, and taken together with the 90-day feeding trial data forms the optimal basis for a comparative assessment of the safety of the GM food.

In June 2007 FSANZ hosted a workshop to discuss the role of animal feeding studies in the safety assessment of genetically modified foods. The findings of the SAFOTEST project were presented and discussed at this workshop. The report from the workshop can be accessed at <http://www.foodstandards.gov.au/search/?keywords=SAFOTEST>. The SAFOTEST approach is also included in ongoing discussions in the self-tasking activity of the Panel on Genetically Modified Organisms of the EFSA.

NOTE: The GM rice lines used in the SAFOTEST project were developed specifically for this project and were not intended for commercial release. Any negative safety issues identified during the project are therefore of no public concern.

Sources:

Knudsen, I. And Poulsen, M. (2007). Comparative safety testing of genetically modified foods in a 90-day rat feeding study design allowing the distinction between primary and secondary effects of the new genetic event. *Regulatory Toxicology and Pharmacology* 49: 53-62.