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Australia New Zealand
Te Mana Kounga Kai – Ahitereiria me Aotearoa

NEW PLANT BREEDING TECHNIQUES

REPORT OF A WORKSHOP HOSTED BY

FOOD STANDARDS AUSTRALIA NEW ZEALAND

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EXECUTIVE SUMMARY

Food Standards Australia New Zealand (FSANZ) hosted a technical workshop to discuss a number of new plant breeding techniques that have come to the attention of regulators. This was the second workshop to be hosted by FSANZ on this topic. A number of scientists with expertise in plant breeding and biotechnology were invited to participate in the workshop.

The objectives of the workshop were to: enhance FSANZ's scientific knowledge and understanding of each of the techniques; and to discuss scientific, technical and regulatory issues, including whether derived food products should be regarded as genetically modified (GM) food. The scientific conclusions of the workshop may constitute a relevant consideration to which FSANZ may have regard when considering applications to amend Standard 1.5.2 – Food produced using Gene Technology in the *Australia New Zealand Food Standards Code*.

The techniques discussed were:

1. **Accelerated breeding following induction of early flowering** – a technique for shortening the flowering time in tree species, to accelerate the breeding process. It involves using a transgenic early flowering plant line as one of the breeding parents. In the final breeding steps, plant lines are selected that have not inherited the early flowering transgene.

2. **Targeted mutagenic techniques** – a range of techniques that have been developed for introducing mutations at specific sites in genomes. This is in contrast to more traditional mutagenic techniques where mutations are random. Depending on how the various targeted techniques are deployed, mutations can either be restricted to one or a few nucleotides or involve the insertion of a new piece of DNA. The specific techniques discussed were:

    a. **transcription activator-like effector nucleases** (**TALENs)** – artificial restriction endonuclease enzymes generated by fusing a transcription activator-like effector DNA binding domain to a non-specific DNA cleavage domain (nuclease).

    b. **type II clustered, regularly interspaced, short palindromic repeats (CRISPR)/Cas systems** – an engineered DNA targeting complex which relies on a small guide RNA in association with an endonuclease (Cas9) to target specific sites in the genome for cleavage.

    c. **meganucleases** - endonucleases with large cleavage sites that can target specific sites in the genome.

    d. **triplex-forming oligonucleotides** - short, single-stranded, synthetic oligonucleotides that are linked to a restriction endonuclease for targeting specific sites in the genome.

3. **Agro-infiltration** – a technique that is primarily used for the transient and localised expression of genes in a plant typically without any integration of the introduced DNA into the plant genome. It involves infiltrating tissues (usually intact leaves) with a liquid suspension of *Agrobacterium* containing the vector. The technique was primarily developed for use as a research tool however is now being used as a production platform for high value proteins, e.g. vaccines.

In relation to **accelerated breeding following induction of early flowering** it was concluded the final food producing lines would be comparable to those developed using a

conventional plant breeding approach. Derived food products should therefore not be regarded as GM food. It would be important from a safety perspective however for the early flowering transgenic parent line to be fully characterised to make it easier to ensure any introduced transgenes have been excluded from the final food-producing lines. This technique is still in the research phase, therefore commercial products are not expected for some time.

For the **targeted mutagenic techniques** it was concluded they are all conceptually similar to zinc finger nuclease technology, which was discussed in the first FSANZ workshop. When used to introduce small changes only, such techniques do not present a significantly greater food safety concern than other forms of mutagenesis. Providing any transgenes have been segregated away from the final food producing lines, derived foods would be similar to food produced using traditional mutagenic techniques. Such foods should therefore not be regarded as GM. When used to introduce a new gene however, the techniques would be equivalent to transgenesis and, as such, any food products should be regarded as GM.

For **Agro-infiltration** it was concluded the technique would have limited applicability to food. As any food products that are produced using this type of expression system will be purified proteins, and the plants in which they are produced will not themselves be used as food, there are no significant food safety concerns. Whether the purified protein products are regarded as GM foods would depend on their use and whether the plants from which they are derived are themselves GM.

Acknowledgement
FSANZ thanks all participants for generously donating their time and for enthusiastically contributing their knowledge and expertise to the discussions.

INTRODUCTION AND BACKGROUND

Food Standards Australia New Zealand (FSANZ) hosted a technical workshop on a number of new plant breeding techniques. This was the second workshop to be hosted by FSANZ on this topic. The purpose of the workshops has been to improve FSANZ's knowledge and understanding of various new breeding techniques and to discuss scientific, technical and regulatory issues surrounding their potential use in commercial agriculture.

The two workshops were initiated following a number of enquiries to FSANZ regarding the regulatory status of products generated from several new plant breeding techniques. In contrast to the techniques used to generate genetically modified (GM) foods that have been assessed and approved to date – which are all derived from transgenic plants – many of the new techniques do not result in final food producing lines that are transgenic. It is therefore not always clear whether derived food products would come within the scope of Standard 1.5.2 – Food produced using Gene Technology in the *Australia New Zealand Food Standards Code* (the Code), and therefore be subject to pre-market safety assessment and approval.

The first workshop, held in May 2012, considered the scientific question of whether foods derived from a number of new plant breeding techniques should be regarded as GM food, or whether they are more like conventional food. The report of the first workshop is available on the FSANZ website (link). The second workshop considered the following additional techniques:

- accelerated breeding following early flowering;
- targeted mutagenesis techniques not discussed in the first workshop; and
- Agro-infiltration.

The scientific conclusions of these workshops may constitute a relevant consideration to which FSANZ may have regard when considering applications to amend Standard 1.5.2 – Food produced using Gene Technology.

A number of scientists with expertise in plant biotechnology and plant breeding were invited to participate in the workshop. They were:

Name	**Position**
Professor Bernard Carroll	School of Chemistry & Molecular Biosciences, University of Queensland
Dr Rob Defeyter	Intellectual Property Manager, CSIRO Plant Industry
Dr Allan Green	Deputy Chief, CSIRO Plant Industry
Dr Roger Hellens[1]	Science Group Leader, Genomics, Plant and Food Research NZ
Professor Peter Langridge	Director and CEO, Australian Centre for Plant Functional Genomics, University of Adelaide
Dr Bill Taylor[2]	Business Development Manager, CSIRO Plant Industry
Professor Peter Waterhouse	School of Molecular Bioscience, University of Sydney

Other workshop participants were staff from FSANZ, the Office of the Gene Technology Regulator, the Australian Government Department of Agriculture, and the New Zealand Ministry for Primary Industries. The workshop was chaired by Professor Peter Langridge, a FSANZ Scientific Fellow.

[1] Dr Hellens is now Professor of Agricultural Biotechnology at the Queensland University of Technology.
[2] Dr Taylor has since retired.

DISCUSSION OF THE TECHNIQUES

Accelerated breeding following induction of early flowering

Overview of the technique

The main objective of this technique is to accelerate the breeding process by shortening the time it takes for a plant to flower (juvenile stage). Some tree species can have long juvenile stages, lasting ten years or more, which means the breeding process can be both time consuming and costly. This is especially the case if new traits are being introduced from wild species, where extensive backcrossing is required to eliminate unwanted traits that are carried over in the process. Shortening the juvenile stage is therefore a very important breeding objective for some species.

A number of different approaches exist for inducing early flowering, including transgenic as well as more conventional approaches[3]. The latter have been used with varying degrees of success but have generally not been able to reduce flowering time to less than twelve months.

Transgenic approaches, on the other hand, have been shown to significantly shorten the flowering time of fruit trees. They primarily involve the over-expression of various genes involved in the flowering pathway. For example, the over-expression of flower-inducing genes such as *LEAFY* (*LFY*), *FRUITFUL* (*FUL*), *APETALA1* (*AP1*) or *FLOWERING LOCUS T* (*FT*) has resulted in significant reductions in flowering times in certain fruit species. RNA interference has also been used successfully to induce early flowering by silencing specific genes involved in flowering repression, e.g. *TERMINAL FLOWER 1-1* (*TFL1-1*), *TERMINAL FLOWER 1-2* (*TFL1-2*).

Many of the over-expressed flowering genes e.g. *FT* and *AP1*, belong to the MADS-box gene family which all encode proteins characterised by a highly conserved DNA-binding domain known as the MADS-box. MADS-box genes are found in animals, fungi and plants and generally encode transcription factors.

The approach to accelerated breeding is to use the early flowering trait to facilitate the production of a number of crossbred generations in the space of a few years. This strategy is particularly useful for introducing single traits from distant wild species and then backcrossing with high quality cultivars to remove any unwanted traits. Using this approach it might be possible to achieve several backcrosses within a decade, which would be a significant acceleration of the breeding process. In the final stages, the transgene is selected against so that only the genes of interest (e.g. a new disease resistance gene), introduced via conventional breeding processes, remain. The breeding process therefore commences with a transgenic plant as one of the parental lines but the final food producing lines will not be transgenic.

One of the best known examples of using this approach is the work by Flachowsky *et al.* (2011)[4] with transgenic apples over-expressing the *BpMADS4* gene from silver birch. The *BpMADS4* gene is a homologue of the *FUL* gene from *A. thaliana* and a member of the MADS-box gene family. A single transgenic line was selected which flowered within a few months. This line is being used in a cross breeding programme to introgress fire blight

[3] E.g. selection of naturally precocious breeding stocks, root pruning and girdling, grafting onto specialised rootstocks, application of growth regulators, imposition of stress, and intensive management of plant nutrition.

[4] Flachowsky H, Le Roux P-M, Peil A, Patocchi A, Richter K, Hanke M-V (2011) Application of a high-speed breeding technology to apple (*Malus* x *domestica*) based on transgenic early flowering plants and marker-assisted selection. New Phytologist 192:364-377

resistance from wild apple (*Malus fusca*) and combine that trait with several resistance genes to apple scab and powdery mildew. Transgenic seedlings carrying the combined resistance traits will then be crossed with 'Golden Delicious' to continue elimination of unwanted traits acquired from the wild species. During the backcrossing process with other elite cultivars non-transgenic, multi-resistant seedlings can be selected which can be further used in a classic breeding programme to obtain the final commercial lines. While this remains a lengthy and complex process, it is nevertheless significantly faster than the classic plant breeding approach.

While this technique is mainly being exploited for tree breeding, broader applications are also being considered. For example, in temperate cereals to convert winter to spring genotypes as a way of allowing multiple generations per year.

Discussion

The main points from the discussion are summarised below:

- In terms of any changes, both intended and unintended, arising from the inserted transgene and the early flowering phenotype, these will be confined to the early generations as the process results in the transgene being segregated away during the latter stages of the breeding process.

- The most important thing from a safety perspective would be for the starting transgenic line to be fully characterised so that the number of copies of the transgene in the original event are known. It would then be reasonably straightforward to ensure all insertions have been excluded from the final food-producing lines.

- Once any introduced transgenes have been segregated away, any changes associated with those transgenes should no longer be present in the final food producing lines or products. The final food producing lines and derived food products would therefore be comparable to those derived using a conventional plant breeding approach.

- While this technique would be successful in accelerating the time it takes to do the initial crosses, a significant amount of time would still be required before an acceptable commercial product might be developed. Commercial products are therefore not expected for some time.

- Some parallels exist between the early flowering technique and some of the other techniques discussed in the first workshop such as seed production technology and reverse breeding. In all three cases, a transgenic plant line is used in the early stages to facilitate the breeding process, but the final food-producing lines are non-GM.

Conclusion

Providing the breeding process results in complete removal of the early flowering transgenes, the final food producing lines will not be transgenic. Food products derived using this technique should therefore not be regarded as GM food.

Targeted mutagenic techniques

Overview of the techniques

The techniques discussed were:

- transcription activator-like effector nucleases (TALENs)
- type II clustered, regularly interspaced, short palindromic repeats (CRISPR)/Cas systems
- meganucleases
- triple helix-forming oligonucleotides (TFO)

These techniques described in Table 1 below, which also includes zinc finger nuclease (ZFN) technology discussed in the first FSANZ workshop, can all be regarded as different tools for achieving the same objective – the introduction of a double stranded break (DSB) at a specific site in the plant genome. Once the DSB has been made, the options for introducing a mutation at the break site are the same irrespective of the technique used.

Mutations are introduced using the cells own repair pathways - either non-homologous end joining (NHEJ) or homology-directed repair (HDR). The two repair pathways differ in their fidelity and template requirements, with NHEJ being the dominant repair pathway in plants. In NHEJ, the cleaved ends are modified then directly ligated using little or no sequence homology. In HDR, a homologous sequence is required to guide the repair. In normal circumstances, a homologous repair template is provided by the sister chromatid, leading to the faithful restoration of the original sequence. However, to introduce a specific change at the break site, an exogenous DNA template can be provided. The resultant changes can range from point mutations to the insertion of new genes.

In terms of delivery to the plant cell, techniques such as ZFN technology, TALENs and CRISPR/Cas9 typically use either *Agrobacterium*-mediated or protoplast transformation. Where this results in stable, rather than transient, gene expression, the intention is to segregate out the introduced DNA prior to commercialisation. In the case of the meganucleases, because of their stability, these can be directly transferred to the plant cell (e.g. by biolistics or electroporation) or indirectly transferred via mRNA which is then translated in the plant cell. Both delivery methods circumvent the introduction of DNA and its subsequent integration into the host genome. In the case of TFOs, delivery is not possible via *Agrobacterium* transformation and hence relies on techniques such as biolistics, electroporation, polyethylene glycol-mediated transformation and silicon carbide whiskers-mediated transformation.

Table 1: Description of the various targeted mutagenic techniques

Technique	DNA recognition and cleavage	Comments
Zinc finger nuclease (ZFN) technology[5]	DNA recognition is by an engineered array of zinc finger DNA binding domains, each interacting with three nucleotides. Cleavage is by a non-specific endonuclease domain derived from *Fok1*.	ZFN dimers can target up to 36-bp sequences. Adjusting the specificity of ZFNs relies on the shuffling of domains with established triplet specificity.
Transcription activator-like effector nucleases (TALENs)	DNA recognition is by an engineered transcription activator-like effector (TALE) protein consisting of an array of 12 to 26 repeats, each interacting with a single nucleotide. Like ZFN technology, cleavage is by a non-specific endonuclease domain derived from *Fok1*.	The construction of engineered TALENs is challenging but a variety of assembly methods have been developed, including automatable high-throughput techniques. TALENs have been used for targeted mutagenesis in *Arabidopsis*, tobacco, rice and *Brachypodium* (bunch grasses) and are predicted to be extended in the near future to crops such as soybean, potato and canola.
Clustered, regularly interspaced, short palindromic repeats (CRISPR)/CRISPR-associated (Cas) system	DNA recognition is by an artificial single guide RNA (sgRNA) that has embedded within it a sequence complementary to the target sequence. Cleavage is by a type II CRISPR-associated nuclease (Cas9).	The RNA-guided Cas9 can function in a variety of cells and organisms to introduce DSBs at specific sites. Because the CRISPR/Cas9 system relies on duplex or triplex formation between RNA and DNA it potentially has much higher specificity than protein-based systems. Only a short fragment in the sgRNA needs to be designed to target the desired sequence in contrast to ZFN technology or TALENs where complex manipulations of proteins are required. CRISPR/Cas systems have so far been used for targeted mutagenesis in *Arabidopsis*, tobacco, sorghum and rice.
Meganucleases	DNA recognition is by a DNA binding domain which binds to very long (12 – 40 bp) and sometimes asymmetric recognition sequences. In meganucleases, the DNA binding domain is not clearly separated from the catalytic (cleavage) domain. This makes meganucleases difficult to engineer for targeting specific DNA sequences.	Re-designed meganucleases with tailored substrate specificity have the advantage of being extremely specific as well as very stable proteins. A re-designed meganuclease from *Chlamydomonas reinhardti* chloroplast(1-*Cre*I)has been used to successfully introduce mutations into the *liguleless1* locus of maize (Gao et al 2010)[6].
Triplex-forming oligonucleotides (TFOs)	DNA recognition is by short, triple-helix forming synthetic oligonucleotides which are conjugated to a restriction endonuclease.	Application of this approach is mainly hampered by the restriction of triple-helix formation to DNA fragments with strands composed of either purines or pyrimidines. The overall efficiency of the TFO technique is low but the high specificity (as a result of nucleic acid-DNA recognition) ensures off-target effects are minimal.

[5] ZFN was discussed in detail at the first FSANZ workshop on New Plant Breeding Techniques and is included here for comparison.
[6] Gao et al (2010) Heritable targeted mutagenesis in maize using a designed endonuclease. The Plant Journal 61: 176-187

Discussion

The main points from the discussion are summarised below:

- Most interest is centred on developing the ZFN, TALENS and CRISPR/Cas9 systems. There is little interest in pursuing the meganuclease or TFO techniques for targeted mutagenesis in plants.

- One of the main issues to consider in relation to the various techniques is their specificity, in particular the possibility of unintended (off-target) effects. In general, the longer the recognition site in the DNA, the greater the specificity. In the case of ZFN technology however a long recognition site would require the engineering of a very large ZFN, which would be quite challenging. Whereas for TALENS, the addition of extra repeats to accommodate a longer recognition site would probably be much easier. The main problem with recognition by protein/DNA binding is that the current understanding of the interaction is still quite limited. In contrast, knowledge of how nucleic acids bind is well developed and the rules for interaction are quite precise. A RNA/DNA interaction is probably more rigorous than a DNA/DNA interaction. Because it relies on nucleic acid binding, rather than protein-DNA binding, for recognition, the CRISPR/Cas9 system will have greater specificity compared to the ZFN and TALENS systems.

- Stable transformation remains the preferred method for delivering the nuclease constructs to the cell, although transient delivery systems are also being investigated. Where stable transformation is used, the aim is to segregate away the introduced construct once the desired mutation has been obtained so that no transgene remains in the final food producing line.

- While both NHEJ and HDR are the main repair mechanisms for DSBs in cells, the occurrence of natural HDR in plant cells is actually very rare (possibly one in a million). It is likely, therefore, that even when a template is provided to direct repair of a DSB, the repair mechanism used by the plant cell will be NHEJ, rather than HDR. NHEJ repair is typically accurate and efficient but is prone to the formation of sporadic unfaithful repair products, such as small deletions or insertions, frequently leading to gene disruption.

- The same general principles and issues which were considered in the first workshop in relation to foods derived using the various ZFN techniques also apply to these other targeted mutagenic techniques. It was noted that the specificity of the techniques is improving all the time which should further limit any potential for off-target effects.

Conclusion

The mutagenic techniques discussed at this second workshop are conceptually similar to ZFN technology. When used to introduce small changes, such techniques do not present a significantly greater food safety concern than other forms of mutagenesis. Providing the introduced DNA has been segregated away from the final food producing lines, food derived from plants modified using these techniques would be similar to food produced using traditional mutagenic techniques, and should therefore not be regarded as GM food. When used to introduce a new gene however, the techniques would be equivalent to transgenesis and, as such, any food products should be regarded as GM.

Agro-infiltration for transient expression

Overview of the technique

Agro-infiltration is a technique that was primarily developed as a research tool for evaluating the activity and function of candidate genes and promoters without the need to stably transform plants. The technique involves cloning the genes/promoters of interest into a specific vector which is then transformed into *Agrobacterium*. The *Agrobacterium*, in a liquid suspension is infiltrated into the intercellular space of the plant tissue where it transfers the gene of interest to the plant cell. Expression is usually only transient and occurs without any integration of the introduced DNA into the plant genome because the target cells are usually not dividing. That is, the plants are not transgenic plants. Agro-infiltration can however also be used to develop transgenic plants if, for example, flowers that contain germ line cells are the target tissue for infiltration. Agro-infiltration can be performed with a variety of expression vectors, including non-viral or plant virus-based vectors.

The most common tissue that is used as a target for infiltration is leaf. A suspension of the bacterium can be infiltrated into the leaf of an intact plant, or alternatively leaf discs, leaves or whole plants can be submerged in the bacterial suspension under vacuum. The technique has been used successfully for a variety of plants including tobacco, *Arabidopsis*, grape, pea and flax.

While primarily used as a research tool, the technique is also being developed as a production platform for high value products such as pharmaceutical proteins (e.g. vaccines), which are normally produced in cell culture. This type of technology could also potentially be used to produce proteins, particularly enzymes, used in food processing.

The key features of Agro-infiltration as a transient expression system are:

- plant material is destroyed or harvested at the end of the production process
- production is almost always contained within a glasshouse or laboratory
- it is rapid, scaleable with very high expression levels being achieved
- it is relatively inexpensive and easy to use

Discussion

There was considerable discussion around whether the system would actually be used for food substances that are low in value compared to pharmaceutical products. It was noted that in the area of enzyme production, bacterial fermentation systems are already very cost-effective. It was also suggested that stable transformation would be a more efficient system than transient expression for industrial-scale production of food substances.

The key points from the discussion were:

- From the food perspective, the most likely substances to be produced will be food processing enzymes or food additives and potentially also protein supplements. It was noted that food processing enzymes and food additives are not regarded as food, and are regulated under separate Standards in the Code, irrespective of whether or not they have been produced using GM techniques.
- For food products such as protein supplements, whether they are regarded as GM food would depend on whether the expression vector becomes stably integrated into

the plant genome. Such integration events may occur at low frequency in the infiltrated area, however if only somatic (non-germ line) cells are involved the integrated DNA will not be inherited in the next generation.

- As the food products that are produced using this type of expression system will be purified proteins, and the plants in which they are produced will not be used as food, the technique does not raise a potential food safety concern.

Conclusion

The technique is considered to have low applicability to food although it could be envisaged as a potential production platform for proteins to be used as food. In this case, it does not raise any food safety concerns. Whether any purified protein products are regarded as GM foods would depend on their use and whether the plants from which they are derived are themselves GM.

New Plant Breeding Techniques

Report of a Workshop hosted by
Food Standards Australia New Zealand

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EXECUTIVE SUMMARY

Food Standards Australia New Zealand (FSANZ) convened an expert scientific panel to provide advice on a number of new plant breeding techniques that have come to the attention of regulators. **Appendix 1** provides details of the membership of the panel. The techniques discussed were:

1. **Pioneer Hi-Bred International's proprietary seed production technology (SPT)** - developed for use in corn to improve the efficiency of hybrid seed production. It involves using a genetically modified (GM) plant line to propagate a male-sterile plant line which is then used as one of the parents to produce hybrid seed. The genetic modification is not inherited by the hybrid plant line.

2. **Reverse breeding** - a novel plant breeding technique that involves suppressing meiotic recombination in order to recreate homozygous parental lines that, once hybridised, reconstitute the composition of an elite heterozygous plant without the need for backcrossing or selection.

3. **Cisgenesis and intragenesis** - involve transferring a new gene into the genome of a plant using gene technology. In both cases the gene is derived from either the same or a cross-compatible species.

4. **GM rootstock grafting** - involves grafting the vegetative part of a non-GM plant (the scion) onto the rootstock of a GM plant to create a chimeric plant that shares a single vascular system.

5. **Oligo-directed mutagenesis (ODM)** - involves the use of synthetic oligonucleotides to introduce small, site-specific mutations into the plant genome.

6. **Zinc-finger nuclease (ZFN) technology** - involves the use of an engineered zinc finger nuclease to introduce site-specific mutations into the plant genome. Depending on the type of ZFN technology deployed, mutations can either be restricted to one or a few nucleotides or involve the insertion of a new piece of DNA.

The objectives of the workshop were to: enhance FSANZ's scientific knowledge and understanding of each of the techniques; and provide scientific advice on the nature of derived food products. It was not the role of the panel to make a legal determination as to whether the techniques or their derived food products would come within the definition of 'food produced using gene technology' in Standard 1.5.2 of the *Australia New Zealand Food Standards Code*. However, the expert panel were asked to provide their scientific opinion on whether derived food products should be regarded as GM food.

As a result of the panel discussion, the techniques were grouped into three categories. **Category 1** comprises cisgenesis/intragenesis, targeted gene addition or replacement using ZFN technology, and GM rootstock grafting. It was the view of the panel that foods produced using these techniques should be regarded as GM food and undergo premarket safety assessment. In the case of cisgenesis/intragenesis and targeted gene addition or replacement using ZFN technology the derived food would be similar to that produced using standard transgenic techniques. Consideration of GM rootstock grafting was more complicated because food produced by a non-GM scion grafted onto a GM rootstock would not contain any introduced DNA. However, it may contain novel RNA and/or protein as a result of the genetic modification to the rootstock. Depending on the genetic modification, the food may also have altered composition or other characteristics. The panel did however note the following:

- in the case of cisgenesis and intragenesis, a simplified form of food safety assessment may be warranted because the transferred genes will be derived from the same or a closely related species which is likely to be commonly used as food and have a history of safe use;

- in the case of GM rootstock grafting, the majority of foods will not contain any novel genetic material or have altered characteristics and therefore should only require a simplified food safety assessment.

Category 2 comprises techniques used for targeted mutagenesis, including ODM and ZFN technology. It was the view of the panel that changes introduced using such techniques would be typically small and definable and have predictable outcomes. Such techniques would therefore be similar to traditional mutagenic techniques used in conventional plant breeding and food derived from these plants should not be regarded as GM food.

Category 3 comprises techniques which involve the use of gene technology at an early stage that is separate from the final plant breeding process. The techniques in this category include SPT and reverse breeding. Although not specifically discussed, the panel noted that accelerated breeding following induction of early flowering using gene technology could also be included in this category. In the case of SPT the panel was of the view that food produced using this technique should not be regarded as GM food as a genetic separation exists between the early GM ancestor (known as the GM maintainer line) and the non-GM parents of the final food-producing line, which does not contain the genetic modification. The panel considered however that it would be useful to have more information confirming the reliability of the sorting technique for indicating the presence or absence of the introduced genes as well as general compositional analysis confirming the equivalence of an F1 hybrid produced via SPT with a standard F1 hybrid.

While there are clear parallels with SPT, the panel did not consider they could reach firm conclusions about reverse breeding because insufficient technical detail was available on how transgene-free end products are produced, as well as the reliability of the process overall. They noted however that there did not appear to be any particular hazards associated with the GM component of the technique. The panel also considered it would be helpful to develop some criteria for distinguishing techniques such as SPT, accelerated breeding and reverse breeding from those where the final food-producing lines are clearly GM and also for ensuring that a complete barrier/genetic separation exists between the early GM breeding lines and the non-GM food-producing lines.

Acknowledgement
FSANZ greatly appreciates the enthusiastic approach of the expert panel to this task and their contribution of knowledge and expertise to this work.

Contents

BACKGROUND

All genetically modified (GM) foods in Australia and New Zealand are subject to approval in Standard 1.5.2 – Food produced using Gene Technology under the *Australia New Zealand Food Standards Code* (the Code). Approval is contingent on completion of a food safety assessment.

The original intent of the standard, which came into force in 1999, was to ensure that food produced using recombinant DNA technology was referred to Food Standards Australia New Zealand (FSANZ) for pre-market approval. Food produced using conventional breeding techniques was intended to be excluded from the standard. At the time, conventional plant breeding techniques were considered to include "traditional cross-breeding, mutagenic techniques, and cell culture techniques such as hybridisation or protoplast fusion"[1].

During the thirteen years in which the standard has operated, all GM food that has been submitted to FSANZ for assessment and approval has been derived from transgenic[2] plants. During 2011 FSANZ received a number of enquiries from researchers and industry about the regulatory status of various plant breeding techniques developed more recently. This generated significant debate within FSANZ as it was not immediately clear whether (i) such techniques would be captured by the current definitions in Standard 1.5.2, or (ii) if they were captured, whether that would be scientifically appropriate and consistent with the original intent of the Standard. Similarly, it was necessary to consider whether any new technique not captured would raise food safety concerns.

To assist FSANZ to address these issues, an expert panel was convened and asked to provide scientific advice in relation to six techniques at a closed technical workshop held on 11 May 2012. The objectives of the workshop were to: enhance FSANZ's scientific knowledge and understanding of each of the techniques; and provide scientific advice on the nature of derived food products. It was not the role of the panel to make a legal determination as to whether the techniques or their derived food products would come within the definition of 'food produced using gene technology' in Standard 1.5.2 of the Code. However, members of the expert panel were asked to provide their scientific opinion on whether derived food products should be regarded as GM food.

The expert panel included research scientists with expertise in plant biotechnology and plant breeding as well as familiarity with GM food safety assessment and regulation. Membership of the expert panel is shown in **Appendix 1**. Other workshop participants were from FSANZ, the Office of the Gene Technology Regulator (OGTR) and the New Zealand Environmental Protection Authority (NZEPA). The workshop was chaired by Professor Peter Langridge, Director and CEO, Australian Centre for Plant Functional Genomics, University of Adelaide.

The workshop consisted of a presentation by each panel member on a specific plant breeding technique, followed by questions and discussion with participants. The techniques selected for discussion were: Pioneer Hi-Bred International's proprietary seed production technology, reverse breeding, cisgenesis and intragenesis, GM rootstock grafting, oligo-directed mutagenesis, and zinc-finger nuclease technology. The workshop considered a number of key questions for each technique. The key questions focussed on: the nature of the changes introduced using each technique; whether introduced changes were present in the final food producing line and derived food products; and the potential for unintended effects[3].

[1] ANZFA (1998) P97 Inquiry Report: Food produced using gene technology.
[2] Where 'transgenic' means containing genes or genetic material transferred from another species
[3] It is noted that the occurrence of unintended effects is an inherent and general phenomenon that can also occur in conventional breeding. Such effects, should they occur, do not necessarily give rise to food safety concerns.

DISCUSSION OF THE TECHNIQUES

Seed Production Technology

Overview of the technique

Seed production technology (SPT) is a proprietary technique developed by Pioneer Hi-Bred International (Pioneer) to facilitate the production of hybrid seed in corn. A hybrid is the result of a cross between two genetically distinct inbred plant lines. Inbred parent lines are produced by successive rounds of self-pollination and are often described as pure breeding lines because every genetic locus is homozygous (ie two identical forms of the same gene). The progeny obtained from the self-pollination of inbred lines will thus be identical to the parents. When the right combination of inbred parent lines is selected, a first generation hybrid (referred to as the F1 hybrid) will exhibit greater yield, uniformity and vigour than either of the parents and in some cases may also exhibit greater disease and insect resistance. This phenomenon is referred to as 'hybrid vigour'. The use of F1 hybrids has significantly improved corn production and is also being widely used in number of other cropping systems eg other cereal crops, cotton, canola, and various horticulture crops.

For hybrid production to be successful it requires the prevention of self-pollination in one of the inbred parent lines (referred to as the 'male sterile' or 'female' line), to ensure pollination is only by the other (inbred) parent line. The most common method used commercially to prevent self pollination is emasculation through the mechanical removal of the male flowers. In corn, the male flowers (tassels), located at the top most part of the plant, are removed by a laborious process called 'detasseling'. While effective, detasseling is not completely reliable[4] and in some cases can result in significant reductions in seed yield due to mechanical damage to the plants. The use of plant lines that are genetically male sterile is an alternative approach that can be used however a major limitation is that propagation of male-sterile lines is time consuming and complex as self-pollination cannot be used.

SPT involves a GM process to facilitate the maintenance of male-sterile inbred lines for use in hybrid seed production. Although initially developed for use in corn, SPT is potentially applicable to a number of other species. For example, a similar system in rice is close to commercialisation and the Australian Centre for Plant Functional Genomics is currently investigating its use in wheat.

In contrast to the mechanical method of creating a male-sterile (female) inbred line, the SPT system uses GM methods to enable the male sterile line to be propagated by self-pollination. This is achieved by transforming a male sterile inbred (female) line (*ms45/ms45*) with three genes: a fertility restorer gene (*Ms45*); an anther-specific amylase gene (*zm-aa1*); and a seed-specific fluorescent colour marker gene (*DsRed2*). The resulting line, possessing single copies of all three genes, is known as the GM maintainer line. Expression of a single copy of the fertility restorer (*Ms45*) gene in the *ms45/ms45* (homozygous recessive) genetic background restores male fertility and enables pollen production by the GM maintainer line.

However, expression of the amylase gene in the anther results in the depletion of starch which deprives the pollen of the energy reserves it needs for successful pollen germination and fertilisation. This ensures that any pollen containing the transgenes (50%) will be infertile. The remaining 50% of the pollen, which lacks the amylase and other transgenes, will be fertile.

[4] Some plants may escape detasseling or new tassels may emerge after the detasseling process.

The fluorescent colour marker is linked to the other two transgenes and confers a pinkish red phenotype (visible by eye) to any seed expressing the SPT cassette. The DsRed2 protein also emits a strong red fluorescence under appropriate illumination so any seeds containing the SPT gene cassette can be readily identified and separated from progeny seeds that do not contain the SPT gene cassette. Self-pollination of the GM maintainer line therefore produces a 1:1 ratio of two different types of seed: (i) yellow seed, which is non-transgenic; and (ii) pink/red seed, which is transgenic (Figure 1). The seeds are subjected to a high-speed fluorescence sorting process to separate the transgenic seed from non-transgenic seed.

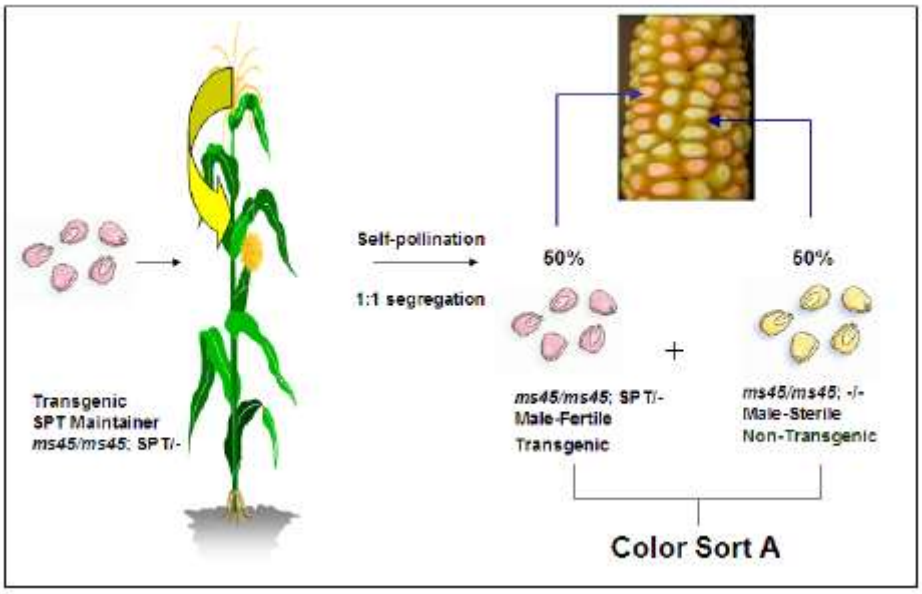

Figure 1: Propagation of the GM maintainer line via self-pollination (Source: Pioneer).

Propagation of the non-transgenic male-sterile (female) inbred parent line involves planting the GM maintainer line (*ms45/ms45*; *Ms45*/-) (red seed) next to rows of the non-transgenic female inbred line (*ms45/ms45*). Fertile (non-transgenic) pollen from the GM maintainer line will cross pollinate and fertilise the non-transgenic male-sterile (female) inbred line, which will produce only yellow coloured (non-transgenic) seed (Figure 2). Several billion seeds have been screened using the fluorescence sorting process and no transgenic seeds have passed through undetected.

The yellow (non-transgenic) male sterile seed is then used as the female parent in commercial hybrid seed production which involves sowing it alongside the seed from a non-transgenic male inbred line – the male inbred line will pollinate the non-transgenic male-sterile (female) inbred line which will produce non-transgenic F1 seed, which is then subsequently sold to growers for food/feed production.

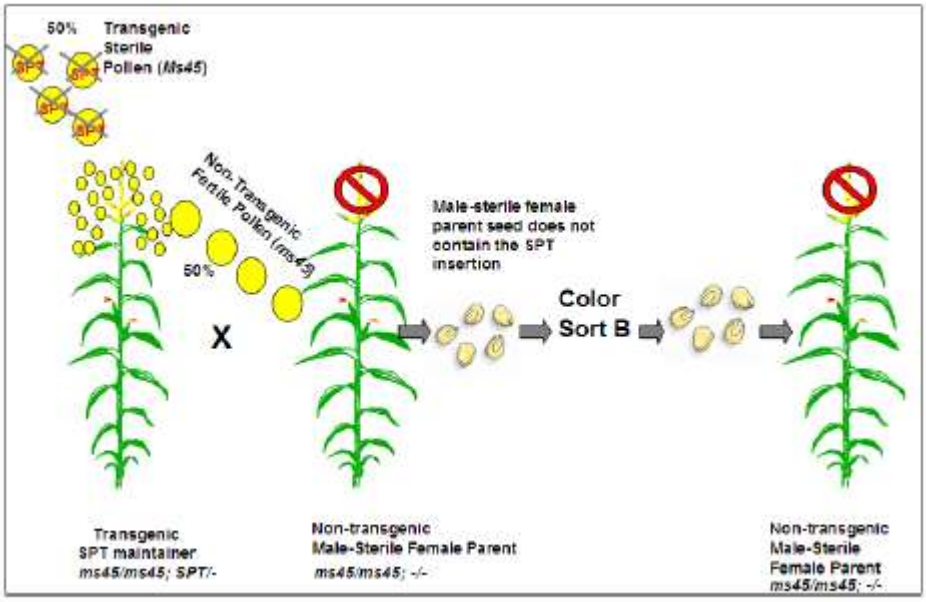

Figure 2: Propagation of the non-transgenic male-sterile female parent line (Source: Pioneer)

Key points:

- The GM maintainer line is required for propagation of the non-transgenic male sterile inbred line;
- Neither the inbred parent lines nor the resulting hybrid lines should contain any transgenes;
- The integrity of the colour screening procedure is essential for ensuring that transgenic seed is not used in the subsequent breeding step.

Panel discussion

While the discussion necessarily focussed on the use of SPT in corn, the panel acknowledged that the principle of SPT could be applied to other crop species, particularly cereals (wheat and rice) where there is a need for better hybrid systems and where alternative GM methods have yet to be developed. It was also recognised that in polyploid species (such as wheat) there may be problems with expression of the male sterility trait due to penetrance issues; this would need to be considered for the individual crop.

The discussion focussed on the following points:

- The integrity of the SPT process is dependent on an intact cassette therefore consideration was given to the potential for the cassette to break up and whether this would allow any of the transgenes to "leak" into the food production lines. Inactivation of *DsRed2* would be detected during propagation of the maintainer line and those seeds (yellow) discarded. The only consequence of inactivation of *DsRed2* would be if it occurred in combination with a failure of the amylase gene (*zm-aa1)* which would enable the production of viable pollen containing the transgenes. It was noted however that if there was a double mutation that inactivated both *DsRed2* and *zm-aa1*, this would be

detected well before any of the transgenes could be transmitted to the food production lines.

- Information on how tightly linked the three genes are in the cassette would be useful for confirming the reliability of the fluorescence sorting as an indicator of the presence/absence of all three transgenes. It was, however, noted that, since the maintainer line is screened at each generation, this in itself would limit any build-up of recombinants, and therefore the likelihood of any escapes entering the food supply would be extremely small. It would also be useful to have general compositional information to confirm the equivalence of an F1 produced through the SPT process with an F1 produced conventionally.

- There was agreement, in principle, that the F1 hybrid produced as a result of crossing the SPT inbred female line with a non-GM male inbred line, would be non-transgenic. On a more pragmatic level it was also noted that if food products derived using SPT were regulated as GM foods, it would be impractical, if not impossible, to detect the F1 hybrid as it would not contain any transgenes.

- If the GM maintainer line was brought into Australia or New Zealand it would require authorisation under the *Gene Technology Act 2000* or the *Hazardous Substances and New Organisms Act 1996*, respectively. The maintainer line is not a commercial product and would not require regulatory approval by FSANZ unless it was to be used for food however the developer may opt to seek food approval of the line as a precautionary measure. It was noted that the United States Food and Drug Administration has already assessed the potential toxicity of the colour marker DsRed2 and the ZM-AA1 amylase and determined that the presence of these proteins at low levels in the food supply would not lead to safety concerns.

Conclusions

The panel concluded the following:

1. Food produced using SPT should not be regarded as GM food as a genetic separation exists between the early GM ancestor (the GM maintainer line) and the non-GM parents of the final food producing line.

2. It would be useful to have more information on the outcomes of any assessment of the likelihood and frequency of the breakup of the cassette as well as general compositional analyses confirming the equivalence of an F1 produced via SPT with a standard F1.

3. If the SPT system were to be deployed in crops that were to be grown in either Australia or New Zealand, the GM maintainer line would require authorisation for environmental release. The developer may wish to apply for food approval of the GM maintainer line used in the SPT process in order to ensure the safety of any transgenic seed that may inadvertently enter the food supply.

Reverse Breeding

Overview of the technique

The conventional way of making elite hybrids and capitalising on hybrid vigour is by crossing selected inbred parental lines. The parental lines can be propagated indefinitely via self fertilisation and crossed together at will to reconstitute the elite heterozygous genotype of the hybrid. The hybrid cannot itself be propagated sexually via seed without eventually losing its heterozygosity and thus its hybrid vigour. In some cases elite or high performing heterozygous lines that have undefined parentage may be identified in a breeding programme. Reverse breeding is a technique that enables the breeder to go backwards from such an individual to directly recreate the homozygous parental lines that created it – essentially allowing mass production through seed of an elite heterozygous genotype.

The reverse breeding process begins with the production of gametes (haploid microspores[5]) from the heterozygous starting plant in which meiotic recombination has been suppressed.

A range of techniques can be used to suppress recombination:

- chemical treatment;
- endogenous mutations – a number have been described for various crop species;
- silencing of key genes involved in recombination events – eg through transient expression of a silencing construct using a viral vector; graft transmission from a transgenic rootstock; or stable transformation of the silencing construct. The typical approach would be to use RNA interference (RNAi) or small interfering RNA (siRNA) constructs to silence the relevant genes;
- introduce genes encoding proteins that are known to interfere with crossing over.

Where GM techniques are being used for the suppression of recombination, the first step of the reverse breeding process would therefore be to transform the elite hybrid with the recombination suppressing construct – usually a hemizygous event. This plant will then give rise to some gametes carrying only non-recombinant (ie parental) chromosomes.

Double haploid (DH) technology is then used to double the chromosomes in the haploid microspores, resulting in a completely homozygous genotype. Homozygous plants can then be regenerated from the DH microspores. The next step is to identify those DH plants which contain complementary sets of chromosomes that will recreate the genotype of the starting plant when crossed – these are the new parental lines. This is done using molecular markers for each of the chromosomes. It is possible to select against the (recombination suppressing) transgene at this stage of the process – although the actual process for doing this is not well described in the literature – so that the selected parental lines do not contain the introduced transgene. Using homozygous parental lines that do not contain the introduced transgene will ensure that the resulting final heterozygous plants are non-transgenic. Once two complementary parental lines are identified, these can be used in a hybrid production system to recreate the heterozygous genotype of the starting plant.

[5] immature pollen grains

Potential limitations with the technique include:

- probably most suitable for vegetatively propagated crops where elite heterozygous varieties exist without inbred parents (where seed propagation will disrupt favourable genetic combinations but may offer some cost/logistical advantages);

- using this technique with F1 hybrids appears to be pointless because the parents will already be known;

- only considered workable for plants with a chromosome number less than 10. When a species with a large number of chromosomes is used, many of the gametes will be unbalanced and either unviable or producing aneuploid[6] individuals with poor vigour. This is because in the absence of crossing over, meiotic events do not occur faithfully (crossing over actually maintains the integrity of the meiotic process), resulting in the formation of a large number of aneuploid gametes. Finding individuals with complementary sets of chromosomes would also be more difficult due to the increased number of potential haploid combinations;

- requires microspore-derived DH technology to be available for the particular crop species;

- requires molecular markers for all of the chromosomes.

Taken together, these limitations point to a fairly narrow range of crops for which this technique might be suitable. At this stage there does not appear to be any real examples or applications in the literature, and the benefits of the technique are not immediately apparent.

Given that any stably introduced transgene would be selected against during the process, unintended effects arising from the technique are considered unlikely. Furthermore there is no rationale for retaining the silencing transgene in the parental lines as its presence is likely to compromise seed production because of irregular gamete formation. Any food products therefore should be equivalent to those derived from a normal hybrid plant that has not gone through the reverse breeding process (unless of course the technique is practised in conjunction with other transgenic modifications).

Panel discussion

On the basis of the available information published so far, the panel did not consider the technique would have widespread application. The types of plants for which this technique might be suitable (based on a chromosome number of 10 or less) include onion, cucumber, barley, sugarbeet, most vegetable Brassicas, lettuce, maize, sorghum, asparagus and cocoa. So far however there are no examples published in the literature. The panel agreed there might be particular advantages to this technique if used in vegetatively propagated species, based purely on cost as it would enable the propagation of an elite heterozygous line to be converted from vegetative to seed propagation (if feasible for that species). The panel could not see any particular advantage for the use of this technique in grain crops.

The main points from the panel discussion are summarised below:

- The panel did not consider that sufficient information is available on the technique, in particular the process used to select against the recombination suppressing transgene so that it is not present in the DH parental lines used to re-create the genotype of the original starting plant. The full technical details of this process do not appear to have

[6] an abnormal number of chromosomes

been adequately described in the literature. It was speculated that for the process to work it would require the use of more than one transgenic event but it was still unclear to the panel how the exact genotype of the starting plant could be reconstituted.

- If the recombination suppressing transgene (if used) is successfully removed in the establishment of the 'parental' lines then the technique can be considered analogous to SPT – that is, it involves the use of gene technology in the development of an early ancestor but the line ultimately used for food production no longer has the transgene present. The panel noted the transgene should leave no genetic 'mark' as the whole chromosome containing the transgene would be selected against. One difference to SPT would be that the use of the transgene, assuming it is successfully removed, would be contained to the laboratory stages – there would be no field production of the transgenic ancestral lines (the double haploids); this would all take place in the glass house with presumably the transgenic plants not taken to the field.

- The panel also briefly discussed accelerated breeding following induction of early flowering as another technique where gene technology can be used in an early breeding stage. For crops that take a long time to flower one of the ways of accelerating the breeding process is to genetically modify them to flower early and thus significantly shorten generation times. For example, apples or plums can be made to flower in 6 months which means shortened breeding cycles but at the end of the breeding process the early flowering trait is selected away so the transgene is no longer present in the lines used for commercial production.

- The panel discussed that it would be possible to develop a guiding principle applicable to these types of technologies (eg SPT, reverse breeding, accelerated breeding following induction of early flowering) that distinguishes them from those techniques where the actual food production lines are transgenic. If that were to be done some guidelines would need to be established regarding the type of information FSANZ would seek to demonstrate that no transgenes are carried through into the field production system. Although the potential risks depend on the different techniques and the genes involved, it may be possible to outline some common criteria for ensuring there is a complete barrier/genetic separation between the early transgenic breeding lines and the non-transgenic food production lines.

- In terms of any safety issues including unintended effects, the panel did not consider there to be any particular hazards associated with the GM component of the technique, although further clarification around removal of the transgene would be necessary.

Conclusions

The panel concluded the following:

1. Parallels exist between reverse breeding and SPT, in that both involve the use of gene technology at an early stage of the breeding process but the final food producing lines are non-transgenic. In the case of reverse breeding, further information is required on how transgene-free end products are produced, as well as the reliability of the removal process. There does not appear to be any particular hazards associated with the GM component of the reverse breeding technique.

2. It would be useful to establish some criteria to assist the decision making process in relation to food derived from non-GM plant lines produced using techniques such as reverse breeding and SPT which involve the use of transgenic lines in earlier generations.

Cisgenesis and Intragenesis

Overview of the technique

Cisgenesis involves the production of a GM plant using donor DNA from the same species or a cross-compatible species. To qualify as cisgenic, the introduced DNA must comprise a natural genomic fragment containing the gene of interest with its own introns as well as regulatory sequences (promoter, terminator). The introduced DNA is in principle free of vector DNA; however the exception is T-DNA border sequences flanking the cisgenic DNA where *Agrobacterium*-mediated transformation has been used. Where microprojectile bombardment using linearised DNA has been used, no extraneous sequences will be present.

Intragenesis also involves the use of donor DNA from the same or a cross-compatible species, however, new combinations of DNA fragments are acceptable, for example using non-native promoters or introducing RNAi.

Both techniques generally involve the use of a selectable marker gene, which will need to be removed unless it is itself a cisgene or intragene. Removal can be by co-transformation and subsequent selection away or via an excisable system. In the latter case there will usually be some residual DNA remaining at the recombination site. However, even if there is no footprint remaining from the transformation process, the introduced gene will be located at a novel locus in the genome (ie the transformation event will be detectable).

Cisgenesis and intragenesis have become possible because of the large amount of genomic information that is now available from genome sequencing of the major crop plants. This means useful genes from within the same species can be identified and transferred to elite cultivars. This is proving to be particularly useful in the development of improved cultivars that are vegetatively propagated. These are crops that are very difficult to breed – it takes a long time to introgress genes from wild species (e.g. bananas, sugarcane, potatoes, strawberries, fruit crops such as apples, avocadoes). The technique enables genes from related species to be introduced without having to go through a difficult and protracted breeding process. Another advantage is that it reduces linkage drag[7] as only a single gene is involved. This also applies to other crops that are easier to breed.

There are a number of examples of cisgenic and intragenic crops that are near commercialisation. Apples with apple scab resistance[8] will be one of the first cisgenic crops to be commercialised. An insect resistance gene has been moved into that species together with all its native regulatory elements plus an excisable selectable marker. These plants have been propagated and are presently undergoing assessment in the United States. Cisgenic potatoes are being developed in the Netherlands, involving stacked genes for late blight resistance. There are also intragenic apples currently being assessed in the United States – these are the so-called “arctic” apples which are non-browning (due to down regulation of the gene encoding polyphenol oxidase).

The European Food Safety Authority (EFSA) genetically modified organism (GMO) panel has concluded that there are similar hazards associated with cisgenic and conventional plants, while novel hazards are associated with intragenic and transgenic plants. While the EFSA GMO panel concluded that the risk assessment applied to transgenic plants could also be applied to cisgenic and intragenic plants they also stated “it can be envisaged that

[7] Linkage drag refers to the phenomenon where other (potentially undesirable) alleles are linked to a desired gene and are incorporated when that gene is introgressed into the genome of a recipient line.
[8] Vanblaene et al (2011) The development of a cisgenic apple plant. J. Biotech. 154: 304-311

on a case by case basis lesser amounts of event specific data are needed for risk assessment of cisgenic and intragenic plants"[9].

Panel discussion

There was a consensus view that cisgenic and intragenic plants are generated by the use of recombinant DNA techniques and food derived from such plants should be regarded as GM food. The discussion then focussed on the public perception of cisgenesis/intragenesis and whether there is any scientific distinction between these techniques and transgenesis which would justify a different regulatory approach.

The main points from the panel discussion are summarised below:

- Various surveys of consumer attitudes indicate that consumers have fewer concerns about cisgenics and intragenics suggesting they are likely to be more accepting of foods produced using these techniques. This should not however influence the scientific assessment that is applied to the product. This will be determined, as always, by the nature of the genetic modification.

- From a technical point of view there is no distinction between cisgenesis, intragenesis and transgenesis as all three techniques involve introducing DNA into a novel site in the genome. The food produced from all three techniques therefore requires pre-market scientific evaluation of safety, and approval under the current Standard.

- The source of the gene (ie whether it is from the same or a different species) could potentially influence the type of safety assessment that would be required. In the case of transgenesis where genes are derived from an unrelated species, some sequences may behave differently in the new host for example if they do not possess a micro RNA site that is important for regulation. Therefore if a gene is derived from an unrelated species and introduced into a heterologous genome its use may raise different questions scientifically than if that gene was derived from the same species, as would be the case for cisgenesis and intragenesis.

- Genes derived from an organism that is commonly used as food and has a history of safe use may require less evidence to establish their safety. For example, allergenicity is unlikely to be an issue, however a full compositional analysis would probably still be necessary, because even in the case of the introduction of another copy of an endogenous gene, its overexpression could have unintended effects. There may therefore be scientific justification for certain types of data to be deemed unnecessary for the safety assessment of cisgenic or intragenic products.

Conclusions

The panel concluded the following:

1. Technically there is no distinction between cisgenesis, intragenesis and transgenesis – all three involve using recombinant DNA techniques to introduce DNA into a novel site in the plant genome.

2. A simplified form of food safety assessment may be warranted for cisgenic and intragenic plants because the transferred genes will be derived from the same or closely related species which are likely to be commonly used as food and have a history of safe use.

[9] EFSA Journal 2012; 10(2): 2561.

GM Rootstock Grafting

Overview of the technique

Grafting is used extensively in many plant breeding programmes and is typically done to modify plant architecture, or to counter biotic and abiotic stresses. The lower part of the plant which contributes roots and support is called the rootstock and the upper part of the plant contributing stems, leaves, flowers and fruit, is called the scion.

The rootstock plus the scion can be regarded as a composite plant which essentially functions as a single organism, with one genotype in the rootstock and another genotype in the scion. A graft union, which consists of a small mass of callus tissue, physically joins the two parts. For many years the graft union was believed to be separate tissue that would act as a selective filter but it is now known there is a vascular connection between the rootstock and scion through which there is one way movement of water and soluble mineral nutrients from roots to leaves via the xylem and two way movement of photosynthates and various macromolecules (RNA and proteins but not DNA) via the phloem. On each side of the graft union all the components of the phloem and xylem are the same and in the same concentration. The graft union is therefore not a selective barrier, nor is it a source of other solutes moving up or down the plant.

Panel discussion

The main points from the panel discussion are summarised below.

- The food produced by a non-GM scion grafted onto a GM rootstock does not contain any modified DNA. However, the evidence indicates there could potentially be novel gene products (such as RNA or proteins) moving from the rootstock into the scion and potentially also into food products (eg fruit). In some cases, the genetic modification to the rootstock may be done to intentionally alter characteristics in the food product. These changes, transmitted via the rootstock, would not be heritable through the seed which is produced in the non-GM scion. Some examples where a genetic modification to the rootstock could alter food characteristics include the use of RNAi to produce non-browning apples or seedless fruit. Most of the applications of GM rootstock grafting would not be of that type however and the presence of novel gene products in the scion, should it occur, would typically not alter the characteristics of the food.

- Although the rootstock and the scion exist together as a composite plant consisting of two different genotypes, the grafted plant essentially functions as a single organism with both the rootstock and the scion being connected by a single vascular system. As a grafted plant can essentially be regarded as a single organism, a plant with a GM rootstock should therefore be regarded as a GMO.

- Where there is transmission of GM material to the food producing parts of the plant or the modification of the rootstock is intended to target the fruit or other edible products derived from the scion, there is a need for regulatory oversight to ensure any potential human health risks associated with consumption of the food are adequately assessed.

- The OGTR, as part of its regulation of GMOs, would undertake an assessment of risks to human health and safety and the environment, part of which involves consultation with FSANZ. While such an assessment provides an avenue to address potential food safety risks associated with grafted plants grown in Australia, it would not necessarily address potential risks from imported foods, including those produced in New Zealand.

- FSANZ could consider a tiered approach to the assessment of foods derived from GM rootstock plants that reflects differences in potential food safety concerns depending on the genetic modification in the rootstock. While a safety assessment would be essential in situations where there is transmission of GM material to the food or where the trait was intended to modify the food, a simplified process could be adopted for the majority of cases where there will be no detectable effects or differences in the food compared with that from a conventional plant.

- FSANZ could consider whether assessment and approval of a particular GM rootstock would, in certain cases, be sufficiently protective of public health and safety to justify excluding the non-GM scion from assessment. This approach would ensure food safety while eliminating the costs associated with unnecessary assessments of multiple non-GM scions. In these cases, the developer of the GM rootstock would be responsible for generating data and seeking regulatory approval.

Conclusions

The panel concluded the following:

1. The grafted plant, consisting of a GM rootstock and a non-GM scion, can essentially be regarded as a single organism. A grafted plant possessing a GM rootstock can therefore be regarded as a GMO.

2. The food produced by a non-GM scion grafted onto a GM rootstock does not contain any modified DNA; however the scion and the food may contain novel gene products (RNA or protein) and have altered characteristics as a result of the genetic modification to the rootstock.

3. The food from a GM rootstock grafted plant should be regarded as GM food and undergo premarket safety assessment. However, a simplified safety assessment would be appropriate in cases where there is no transmission of novel gene products to the food and no altered characteristics as a result of the genetic modification to the rootstock. Depending on the nature of the genetic modification, it may be appropriate to focus the assessment and approval process only on the GM rootstock itself, which would allow grafting of any non-GM scion onto an approved GM rootstock without the need for individual assessment of the composite plant.

Oligo-directed Mutagenesis

Summary of presentation

Oligo-directed mutagenesis (ODM) provides a tool for targeted mutagenesis in plant breeding and can be likened to 'keyhole' breeding that allows small changes (often a single nucleotide) to be made in the genome of the 'parent'. The technique was first developed in yeast and mammalian systems before being used in plants, and has been used for nearly 30 years.

The technology has various forms, the simplest of which involves the introduction of a short piece of chemically synthesised single-stranded DNA (oligonucleotide) of known sequence into a cell which then hybridises to a homologous target DNA sequence in the genome. *Agrobacterium*-mediated transformation cannot be used for introducing the oligonucleotides into plant cells but there are a number of other different mechanisms that can be used, such as: chemical (polyethylene glycol-mediated); electroporation; and biolistics (shooting in gold particles coated in the oligonucleotide).

The introduced oligonucleotide is exactly complementary to the native target sequence except for a single nucleotide change that is to be incorporated into the target sequence. The single mismatch between the introduced oligonucleotide and the target sequence triggers the cell's DNA repair system resulting in a change to the native target sequence. This very simple form of ODM using single-stranded DNA has proven to be relatively inefficient therefore other oligonucleotide templates have been developed comprising mixtures of RNA and single stranded DNA, RNA only, or triple-helix-forming oligonucleotides.

The overall efficiency of the technique is very low, which limits its application, and there has to be a way of selecting for the single base pair change. The two best plant examples are:

- where a non-functional gene is corrected by introducing a single nucleotide change (e.g. chlorsulfuron, green fluorescent protein); and

- Cibus Genetics LLC RTDS technology, which has led to herbicide-tolerant canola that is nearing commercialisation. The conversion of an imidazolinone-sensitive plant to an imidazolinone-tolerant plant was achieved by introducing a single nucleotide change (G to A at nucleotide 1958) into the ALS (acetolactate synthase) gene, which converts a serine to asparagine at amino acid position 653 of the enzyme.

One of the advantages of the technique is that, because of its specificity, off-target effects are minimal. However, in the early literature there were observations of changes on either side of the intended change. Importantly, however, the change that is introduced is really no different from mutations that may occur naturally or with other mutagenesis techniques such as those using ethyl methanesulfonate (EMS) or ionizing irradiation where, as well as the intended change, many other nucleotide changes to the genome can and do occur.

Panel discussion

The main points from the panel discussion are summarised below.

- The technique is limited in its application in plants because it introduces a small specific change into the genome at low efficiency which means ODM has mostly been used to produce mutations that result in a selectable phenotype (e.g. herbicide tolerance). There are however emerging technologies which will make screening for discrete mutations

much easier, both in terms of efficiency as well as cost, therefore the technique may have broader application in plants in the future.

- Most of the published reports in plants describe single point mutations resulting in a single amino acid substitution (eg the Cibus Genetics imidazolinone-tolerant canola), however there appears to be no technical reason why the technique couldn't also be used to delete or insert a specific nucleotide.

- In terms of the mechanism by which mutations are introduced using this technique the evidence indicates that the oligonucleotide carrying the mismatch hybridises to the target site and is then subsequently used as a template by the cell's DNA repair system to introduce the mutation at the target site.

- Given what is known about the mechanism and also that the technique relies directly on a chemically synthesised oligonucleotide molecule, ODM is not considered to be a recombinant-DNA technique.

- In terms of the limited nature of the changes introduced using this technique, the likelihood of unintended effects in the target plant is considered negligible. The technique is very specific compared to other forms of mutagenesis involving chemical treatment or irradiation. The structure of the oligonucleotides used (eg double hairpin) is such that they are not good substrates for non-homologous end joining; therefore they are unlikely to become randomly integrated into other sites in the genome. In addition, the techniques for screening for other insertion sites have been evolving therefore should this occur it could be readily screened for. If the oligonucleotide were to introduce off-target mutations, it's unlikely these would be distinguishable from the background variation that occurs within genomes. Furthermore, in comparison to other mutagenic techniques, such off-target changes would be minimal.

- In theory the technique could be used to introduce more extensive changes, eg by accumulation. While it may be technically possible to use ODM to introduce larger changes, there are other techniques available which would be more efficient. Furthermore, techniques such as chemical mutagenesis and irradiation could also be used to generate more extensive changes by accumulation. It is also possible for this to happen naturally. On this basis ODM is conceptually no different to traditional mutagenesis, although it was noted that insertions would be difficult to achieve using the more traditional forms of mutagenesis.

Conclusions

The Panel concluded the following:

1. ODM is not a recombinant-DNA technique. There are no identified safety concerns associated with the use of ODM, both in terms of the nature and extent of the specific changes that it can introduce to target plants as well as potential unintended effects.

2. Food derived from plants modified using ODM would be similar to that derived using traditional mutagenic techniques, or that occur naturally through spontaneous mutation.

Zinc Finger Nuclease Technology

Overview of the technique

Zinc finger nuclease (ZFN) technology, like ODM, can be used for targeted genome modification in both plants and animals. It relies on a customised ZFN to introduce a double-stranded break (DSB) at a specific genomic location. Subsequent repair of this break leads to the introduction of mutations at the break site.

Other engineered nuclease systems also exist – meganucleases and transcription activator-like effects nucleases (TALENS). Conclusions in relation to ZFN technology will probably also be applicable to these systems.

In terms of design, ZFNs are composed of two separate monomeric proteins, each of which consists of a non-specific endonuclease domain (derived from *Fok1*) linked to an engineered zinc finger DNA binding domain. The *Fok1* endonuclease domain only cleaves as a dimer, a characteristic that is capitalised on to provide target specificity ie the ZFN monomers have to dimerise to be able to cleave DNA.

The zinc finger DNA binding domain is composed of an array of Cys_2 His_2 zinc fingers – usually 3 – 6 per monomer. Each finger recognises 3 nucleotides so that each array can be designed to bind to genomic sequences 9 – 18 nucleotides in length, depending on the number of zinc fingers. There are several different methods available to construct the different zinc finger binding domains. All of these are complex and currently require a time-consuming design process.

In terms of cleavage, each monomer is designed to bind in inverse orientation to the target sequence. The optimal space between the two binding domains is between 5 and 7 nucleotides. It is this binding that brings the two endonuclease domains into close proximity and generates a functional heterodimer that then cleaves the target site. This requires simultaneous expression of the two ZFN monomers in the cell.

Once cleavage has occurred, the DNA is repaired using one of two endogenous DNA repair pathways, either non-homologous end joining (NHEJ), or homologous recombination (HR)/homology directed repair (HDR). The two pathways differ in their fidelity and template requirements, with NHEJ being the dominant repair pathway in both animal and plant cells.

The main characteristics of the two repair pathways are outlined in the following table.

Non-homologous end joining	Homology directed repair
Cleaved ends are directly ligated – no sequence homology required. Regions of micro-homology, eg complementary overhangs, will be used if they exist.	Requires a homologous sequence to guide repair. Homologous repair template normally provided by the sister chromatid – leads to restoration of original sequence. An exogenous DNA template can be provided to introduce specific changes.
Overall, repair is accurate and efficient BUT is prone to the formation of sporadic unfaithful repair products.	Repair is accurate, according to the template.
The frequency of NHEJ-mediated repair of DSBs is generally much higher than HDR-mediated DSB repair. Faithful repair of the ZFN-induced DSB will restore the ZFN target site which means multiple rounds of cleavage and repair can occur in a cell.	

The technique can be used to generate a number of different types of mutations, as follows:

1. ZFN-1 – where NHEJ is used to introduce site-directed random mutations (substitutions, deletions and insertions) involving one or a few base pairs;

2. ZFN-2 – where HDR is used to introduce site-directed specific mutations (substitutions, deletions and insertions) involving one or a few base pairs;

3. ZFN-3 – where HDR is used for targeted gene addition or replacement. ZFN-3 is essentially a site-specific transgenesis.

If more than one DSB is introduced into the genome, NHEJ can also be used to make more extensive mutations such as translocations or major deletions.

In plants the most common method of delivery of ZFN being used currently is stable transformation with a ZFN construct. Transient expression of the ZFN (via a plasmid, viral vector or directly injected mRNA) may also be used and is particularly favoured in animal systems. In plants, expression via extra-chromosomal plasmid DNA has been used and transient expression from plant virus vectors is also being investigated. More recent research has investigated direct delivery of the ZFN protein. In the case of HDR, the template also has to be co-delivered with the ZFN, whatever the delivery method that is used for the ZFN.

Unintended effects primarily arise from off-target or non-specific cleavage. Non-specific cleavage can arise from not having enough zinc finger binding domains, homodimer formation of identical ZFN monomers, or sustained expression of the ZFN in the cell. Non-specific cleavage may lead to cytotoxicity although in many cases the effects will remain silent. Various strategies have been devised for minimising non-specific cleavage. Increasing the number of zinc finger arrays has been shown to improve specificity, with the use of 4-finger arrays now being more common than 3-finger arrays. *FokI* nuclease variants have also been produced that will only cleave as a heterodimer, not a homodimer. The use of inducible, rather than constitutive promoters to stably express the ZFN has also improved the specificity.

Panel discussion

The main points from the panel discussion are summarised below.

- The similarities and differences between ODM and ZFN technology were discussed, noting that unlike ODM, ZFN technology involves site-specific cleavage of the DNA and the use of a recombinant-DNA construct to produce the ZFN. Mechanistically, ZFN-2 is most like ODM in that both rely on an exogenous template to direct the changes that are introduced into the target site. In terms of the nature of the changes that can be introduced, the panel agreed that ODM, ZFN-1 and ZFN-2 are all similar, although it was recognised that there is more specificity associated with ZFN-2 (and ODM). ZFN-3 is distinct from the other forms of ZFN technology because it results in the introduction of a new gene and is therefore equivalent to traditional transgenesis, except the integration is site-specific.

- Technically ZFN-1 and ZFN-2 are the same, except that ZFN-2 is more specific in terms of the changes that are made. As with ODM, ZFN-1 and ZFN-2 do not present a significantly greater concern than traditional forms of random mutagenesis – the changes introduced are small, definable and the outcomes predictable. The panel discussed whether there should be regulation of the number of changes that could be introduced

using this technique. One suggestion involved using two amino acid changes as the cut off, as the likelihood of generating more than this through random mutagenesis was considered extremely low. However it was thought this might be difficult to implement under the current GM food standard and FSANZ application process which does not provide the flexibility to distinguish between different products of the same process. Capture would therefore have to be all or none. It was acknowledged however that it is difficult with ZFN technology because there are potentially a number of different categories of end-products.

- In terms of unintended effects, these mostly relate to the introduction of DSBs at sites other than the intended target site. This has been associated with cytotoxicity, although various strategies have now been developed which have significantly improved the specificity of ZFNs. Non-specific DSBs that prove to be cytotoxic would be self-limiting. The unintended changes that are not cytotoxic would be more of a concern, but, relative to mutagenesis (by chemical treatment or irradiation), ZFN technology is considered a clean procedure.

- Some time was devoted to discussing the various delivery mechanisms and whether this should make any difference in terms of potential safety concerns. In the case where the ZFN is delivered as a protein to the cells, this does not seem to be any different from applying a chemical mutagen such as EMS or exposing the whole plant to radiation. However, if a protein-mediated form of delivery was used for ZFN-3 then this would not negate the fact that the plant is transgenic. Equally, plants stably transformed with a ZFN construct, irrespective of whether it's used for ZFN-1, ZFN-2 or ZFN-3 would also be classified as transgenic. Given the types of ZFN technology in combination with the different delivery methods used, a decision tree might potentially aid in distinguishing between the different categories of end products for the purposes of regulation.

- The panel also discussed the possibility of using ZFN technology, particularly ZFN-3, to create fixed sites (eg 'docking stations') in a genome into which any gene of interest could be inserted. The idea being that once the background has been assessed in terms of unintended effects arising form the insertion event, it would not need to be reassessed again for subsequent applications and instead the assessment would just focus on the inserted gene and any changes associated with its expression. In this situation, the safety assessment would mainly focus on the molecular characterisation and novel protein characterisation (potential allergenicity/toxicity) and possibly a limited compositional analysis, depending on the nature of the introduced gene. However it was noted that if the docking site was used to insert a large number of genes, this might change how the site behaves and therefore the issue of unintended effects would possibly need to be reconsidered.

Conclusions

The panel concluded the following:

1. ZFN-3, which involves introducing a new gene into a specific site in the genome, is equivalent to standard transgenesis.

2. ZFN-1 and ZFN-2 are mutagenic techniques that are conceptually similar to ODM. Such techniques do not present a significantly greater food safety concern than other forms of mutagenesis. The changes introduced using ZFN-1 and ZFN-2 will be small, definable and the outcomes predictable. Food derived from plants modified using ZFN-1 and ZFN-2 would be similar to food produced using traditional mutagenic techniques, and should therefore not be regarded as GM food.

APPENDIX 1: EXPERT PANEL

Chair:

Professor Peter Langridge (Director and CEO, Australian Centre for Plant Functional Genomics, University of Adelaide)

Panel Members:

Dr Paul Brent (Chief Scientist, Food Standards Australia New Zealand)

Distinguished Professor James Dale (Director, Centre for Tropical Crops and Biocommodities, Queensland University of Technology)

Dr Andrew Granger (General Manager, Science, Plant and Food Research NZ)

Dr Allan Green (Deputy Chief, CSIRO Plant Industry)

Dr Roger Hellens (Science Group Leader, Genomics, Plant and Food Research NZ)

Dr Lisa Kelly (Principal Scientist, Food Standards Australia New Zealand)

Present also were: Dr Andrew Bartholomaeus, Dr Mark FitzRoy, Ms Lynda Graf, Dr Janet Gorst, Dr Utz Mueller and Dr Chris Schyvens from Food Standards Australia New Zealand, Dr Michael Dornbusch, Dr Heidi Mitchell, Dr Fiona Murray, Dr Will Tucker and Dr Brian Weir from the Office of the Gene Technology Regulator and Dr Kirsty Allen from the New Zealand Environmental Protection Authority.

Biographies of Panel members

Dr Paul Brent

Dr Brent is Chief Scientist of Food Standards Australia New Zealand. Prior to this, Paul was Manager of Product Safety Standards section, responsible for risk management of a range of product safety standards, including novel foods, irradiated foods, genetically modified foods, food additives and contaminants. Dr Brent has represented FSANZ on GM food issues at several levels, including the Australia New Zealand Food Safety Ministerial Council. Dr Brent has been the Australian delegation leader to the UN/WHO Codex Committee on Food Additives and Contaminants for several years.

Dr Brent obtained his Bachelor of Science at Newcastle University and doctorate in Clinical Pharmacology at the University of Newcastle Medical School prior to working as a Research scientist in basic and clinical pharmacology, neuroscience and biochemistry. Prior to his appointment with FSANZ, Dr Brent worked as a toxicologist at the Therapeutic Goods Administration and has experience in the evaluation of animal and human toxicological data submitted for registration of agricultural, veterinary and industrial chemicals and in support of clinical trials.

Distinguished Professor James Dale

BScAgr (Hons), PhD Syd

Distinguished Professor James Dale is the inaugural Director of the Centre for Tropical Crops and Biocommodities (CTCB) at Queensland University of Technology (QUT) which incorporates Tropical Crop Biotechnology, the Syngenta Centre for Sugarcane Biofuels Development and Sugar Research and Innovation (SRI). The CTCB consists of around 50 researchers as well as more than 25 postgraduate students. Prof Dale has been involved in biotechnology research for more than 30 years with specific interests in (i) the biofortification of bananas, (ii) molecular farming for high value medical proteins, including vaccines, in tobacco and bioethanol from sugarcane, and (iii) the development of disease resistance in genetically modified bananas, papaya and sugarcane. He has also led research and development programs in Vietnam, Thailand, Malaysia, India, Tonga, Fiji, PNG and, in Africa, Uganda, Kenya and Tanzania. Prof Dale has published more than 80 research papers, reviews and book chapters. He an inventor on 9 granted patents or patent applications. He has been a member of the Australian Research Council's College of Experts (2004-2006) and Chair of the Biological Sciences and Biotechnology Panel (2006), and has been a member of the Gene Technology Technical Advisory Committee (GTTAC) of the Office of the Gene Technology Regulator (2001-2007) and the Queensland Biotechnology Advisory Council. Prof Dale is leading a major international Grand Challenges in Global Health Project funded by the Bill and Melinda Gates Foundation to develop biofortified and disease resistant bananas for East Africa. The first Australian GM banana field trials commenced in early 2009 and harvesting commenced in early 2010. There are already multiple lines with greater than the target level of pro-vitamin A. The project was further expanded on 2012 into India through a collaboration with BIRAC. In 2004, Prof Dale was made an Officer in the Order of Australia (AO) for services to agricultural biotechnology.

Dr Andrew Granger

Dr Andrew Granger is the general manager for Breeding and Genomics at the New Zealand Institute for Plant and Food Research and is responsible for leading all its breeding and genomics programmes conducted in a range of crops, including berryfruit, kiwifruit, pipfruit, summerfruit, hops, arable, vegetable and ornamental species. The programmes involve 250 staff and eight research centres across New Zealand, as well as collaborative projects in Spain, Germany, The Netherlands, United States, South Africa, China and Australia. Andrew's key responsibilities are strategic planning, industry relationships and science direction and quality.

Dr Allan Green

Dr Allan Green is Deputy Chief of CSIRO Plant Industry in Canberra, Australia. His background is in plant breeding and genetics, and his main research activities have been in the genetic modification of fatty acid composition in oilseed crops to provide improved nutritional value, enhanced functionality, and novel industrial end uses. He was the founder and leader of the CSIRO plant oil modification research group that has developed and applied conventional and transgenic techniques of biosynthetic pathway manipulation to develop novel oil compositions in canola, cottonseed, linseed and safflower. These include significant reductions in levels of saturates and polyunsaturates to create stable high-oleic cooking oils, and the incorporation of the long-chain PUFAs, EPA and DHA, into seed oils. His current research is focussed on the introduction of new metabolic pathways into oilseeds to develop renewable plant sources of industrial fatty acids to support the emerging bio-based chemicals and materials sectors.

Dr Roger Hellens

Dr Roger Hellens is the Science Group Leader of Genomics at the New Zealand Institute for Plant and Food Research (PFR) and Associate Professor at the University of Otago, New Zealand. The Genomics Group at PFR is comprised of over 80 scientists focused on the genetic basis of production and consumer-driven traits in fruit and vegetables. The Genomics Group works closely with the Breeding Technology and crop-focused Breeding Groups to accelerate the development of premium plant varieties as whole foods and ingredients. Prior to joining PFR in 2001 Dr Hellens gained his PhD and worked at the John Innes Centre, Norwich, United Kingdom, where he worked on transcriptional and post transcriptional gene regulation and the development of research tools for plant genomics research.

Dr Lisa Kelly

Dr Kelly is a Principal Scientist in the Risk Assessment – Chemical Safety and Nutrition Section of Food Standards Australia New Zealand (FSANZ). Her primary area of expertise is in the safety assessment of GM foods, and she also has experience in assessing the safety of chemicals in food (food additives, contaminants, nutrients).

Dr Kelly has considerable international experience in the GM food area - she chaired the OECD Task Force for the Safety of Novel Foods and Feeds from 2003 – 2009 and also led the Australian delegation to the Codex Ad Hoc Intergovernmental Task Force on Foods Derived from Biotechnology, where Australia led the development of a new Codex guideline for the safety assessment of food derived from GM animals. She is also a member of the FAO/WHO Expert Panel on Biotechnology and Food Safety and has participated in two FAO/WHO expert consultations on GM animals.

Dr Kelly received a PhD in molecular plant virology from the Australian National University in 1994 and undertook post-graduate research in plant biotechnology and recombinant antibody technology at CSIRO Plant Industry up until 1997, when she joined FSANZ. Dr Kelly is currently the GM team leader at FSANZ.

Professor Peter Langridge

Professor Langridge is currently the CEO and Director of the Australian Centre for Plant Functional Genomics which was established in 2002 through funding from the Australian Research Council, the GRDC and the South Australian Government. The Centre focuses on tolerance to environmental stresses in wheat and barley. His research has focused on development and application of molecular biology to crop improvement. He served for six years on the Genetic Manipulation Advisory Committee and is currently a member of the Gene Technology Technical Advisory Committee of the Office of the Gene Technology Regulator. He is on the editorial boards of several international journals, member of the Steering Committee of the International Triticeae Mapping Initiative (ITMI) and serves on the Advisory Boards for the European Union BioExpoit and TriticeaeGenome Programs, the National Science Foundation Wheat D-Genome Program in the USA, The Generation Challenge Program of the CGIAR, and the Centre for Integrative Legume Research. In 2010 he chaired Prime Minister's Science, Engineering and Innovation Council who delivered the report 'Australian and Food Security in a Changing World' outlining strategies to address food security globally. Professor Langridge was recently named South Australia's 2011 Scientist of the Year.