NEW PLANT BREEDING TECHNIQUES

REPORT OF A WORKSHOP
HOSTED BY

FOOD STANDARDS AUSTRALIA
NEW ZEALAND

AUGUST 2013

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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.

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.

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[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

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[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 <u>non-transgenic</u>, 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.

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[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.