

ADVISORY COMMITTEE ON NOVEL FOODS AND PROCESSES

TRANSFORMATION-INDUCED MUTATIONS IN GM PLANTS

Issue

The Committee is invited to consider a recent review of mutations induced in plants by the techniques that are used to develop genetically modified lines, and to advise whether this analysis has implications for the current approach to the risk assessment of foods derived from GM crops.

Background

1. The Food Standards Agency has been contacted by the authors of a review published in December in *Biotechnology and Genetic Engineering Reviews* entitled "Transformation-induced Mutations in Transgenic Plants: Analysis and Biosafety Implications" (**Annex 1**). This review describes the presence of unintended genetic changes in plants following the application of techniques used to insert transgenic DNA into the plant genome, namely:
 - Agrobacterium-mediated transformation and
 - Particle bombardment (biolistic transformation)
2. The authors consider both the changes that occur at the site of integration of the transgene and mutations at other locations in the plant genome. It is argued that these changes may be retained in transgenic cultivars and could have consequences for the safety of the final product.
3. The Agency's Chief Scientist has referred this review to the ACNFP for advice. On receipt of the documents, the Secretariat contacted the authors (**Annex 2**) seeking clarification on two points:
 - (a) whether the approach set out in EFSA's¹ guidelines is sufficient to identify the potential risks arising from the unintended genetic changes discussed in their paper.
 - (b) how the transformation-induced genetic changes discussed in their paper compare with natural mutations in terms of likelihood, severity and potential consequences for food safety.

¹ EFSA Guidance document of the Scientific Panel on Genetically Modified Organisms for the risk assessment of genetically modified plants and derived food and feed. May 2006

4. The authors' response is attached at **Annex 3**. On the first point they consider that the analysis of genetic changes should be extended by sequencing 50,000 base pairs either side of the location where transgenic DNA has been inserted, and by comparing this with the sequence at the same locus in untransformed plants. The response at Annex 3 does not comment on the adequacy of the phenotypic and compositional analysis as a mechanism for identifying unintended changes in GM crops and suggests that the current approach excludes hazards that are not related to the predicted function of the transgene. They suggest that the only realistic and effective way to address unanticipated consequences is through animal and clinical studies that examine specific endpoints, in order to assess hazards identified in the course of the formal risk assessment process. However, they do not indicate how the identification of such hazards might be improved, other than by sequencing.
5. On the second point, the authors' view is that, leaving aside genetic modification and wide crosses, plant tissue culture is the most mutagenic form of plant breeding. Although they consider that the bar is set too low for other (non-GM) methods of plant breeding, they argue that this does not provide a rationale for not considering the consequences of unintended genetic changes in GM plants, noting that individual GM events are likely to be grown and consumed on an unusually wide scale [compared with new non-GM varieties].

Committee action needed

6. The Committee is invited to consider the points raised in the attached review (Annex 1) and to advise whether this analysis has any implications for the current approach to the risk assessment of foods derived from GM crops.

**Secretariat
March 2007**

Annexes attached:

Annex 1: Original communication from Dr Jonathan Latham with a copy of the published paper: "Transformation-induced mutations in transgenic plants: Analysis and biosafety implications". *Biotechnology and Genetic Engineering reviews* Vol.23 pp209-234 (2006)

Annex 2: Secretariat request for further information.

Annex 3: Response from Dr Latham

Original communication from Dr Latham

12 January 2007

Dear Andrew Wadge

We have just published the attached paper on transgene integration in *Biotechnology and Genetic Engineering Reviews* Vol 23 December 2006 pp209-237

Title: Transformation-induced Mutations in Transgenic Plants: Analysis and Biosafety Implications.

We bring this paper to your attention because this review is the first peer-reviewed and thoroughly comprehensive analysis of the characteristics of transgene integration and other genetic changes associated with plant transformation as it is used commercially.

In this paper we suggest that the genetic changes associated with plant transformation are neglected biosafety risks of transgenic plants which have a high probability of leading to biological consequences. The paper argues that this is because these changes are much more numerous than commonly supposed (more so even than conventional tissue culture), they are genetically more complex than commonly supposed and often inseparable from the transgenic phenotype. These characteristics make the genetic alterations associated with plant transformation much more significant than those associated with any other known method of plant breeding.

Following from these conclusions the paper makes important recommendations: 1) for improving the safety of transgenic varieties at the point at which they are presented for regulatory approval 2) for further research and 3) for improving the focus and effectiveness of biosafety approval procedures.

The paper for example points out that most regulatory analysis of insertion sites is inadequate for detecting common and potentially significant characteristics of transgene insertion events.

I would appreciate it if you would confirm receipt of this email and I would be interested to hear your comments.

Please would you also pass this paper to ACNFP and ACRE since this paper is relevant to both their work

yours sincerely
Jonathan Latham

Secretariat request for further information

6 February 2007

Dear Dr Latham,

I have been passed a copy of your email of 12 January to Andrew Wadge and I plan to include this as an item on the agenda of a forthcoming ACNFP meeting.

Before doing this, I would be grateful if you could expand upon two issues arising from your review paper.

(a) Your paper refers to "regulators" in general and does not differentiate between the approaches taken in different countries and regions. As you will be aware, risk assessments in the EU are conducted by the European Food Safety Authority. EFSA's guidelines for the assessment of foods derived from GM plants state that the assessment should include both intended and unintended effects of the genetic modification. The guidelines describe the genetic and compositional information that is needed in order to assess risks arising from unintended effects.

The current EU risk assessment strategy is not specifically mentioned in your review and I would welcome your views on whether the approach set out in EFSA's guidelines is sufficient to identify the potential risks arising from the unintended genetic changes that are discussed in your paper. If you consider that it is not, can you suggest how the current approach could be improved?

(b) The plant genome is not set in stone and naturally undergoes changes, for example as a result of spontaneous mutations and rearrangements, or in response to environmental factors. From your investigations, are you able to make any comparison between natural and transformation-induced genetic changes in terms of likelihood, severity and potential consequences for food safety?

I would be grateful if you could reply by 28 February, so that this item can be included on the Agenda of the 22 March meeting.

Response from Dr Latham

26 February 2007

Dear Dr Lawrie

Thank you for your email requesting clarification of our suggestions. Below, I have outlined our thinking in some detail since, judging from the scientific literature, it departs in a number of ways from many published views. I have made some suggestions for improvement and these are in bold in answer to part a).

Your question has two parts, although here I have reversed their order since part b is a useful introduction to part a:

part (b) The context and significance of transformation-induced mutations:

As you pointed out the plant genome is not set in stone, there is natural variation (which itself differs greatly between species-with maize having a particularly unstable genome), and human-induced variation.

Goals in risk assessment of plant breeding

Firstly, we would like to note that risk assessments depend ultimately on their fundamental goals. In the context of our paper, it is typically possible, if one casts the net wide enough, to find a procedure that is in use somewhere that is 'worse' in some way than the novel one which is under consideration. Thus, it has been common for commentators to call attention to relatively rare methods of plant breeding (such as tissue culture) and to define these as the appropriate baseline for comparison.

We do not however recommend this as a useful or constructive risk assessment practice because it runs the risk of tolerating or even initiating a 'race to the bottom', which contradicts a fundamental purpose of risk assessment which is to incentivise improvement and to contribute to safety.

Mutations and crop breeding

Our overall view is that, leaving aside GM and wide crosses, the most mutagenic form of plant breeding is almost certainly tissue culture. There is not the space to go into all the data here and so I will summarise our understanding briefly below.

Induced Mutations

Chemical mutagenesis and irradiation do introduce mutations. It is known from studies of *Arabidopsis* that they normally introduce point mutations (eg Greene et al 2003 Genetics 164:731-740). In the few crop species from which alleles generated artificially

have been isolated and sequenced, point mutations also seem to be the norm (eg the short stemmed green revolution rice Calrose76; Spielmeier et al (2002) PNAS 99:9043-9048).

Tissue Culture and Plant Transformation

Tissue culture divides into two types: plant propagation, which is common, normally considered non-mutagenic and involves no dedifferentiation step; and tissue culture proper, which is highly mutagenic and used much less. The mutagenicity of the latter is widely thought (though not proven) to correlate with time in culture and stress more generally. Thus addition of a plant transformation step increases the time spent in culture but also adds bacterial infection, bombardment and sometimes known or suspected chemical toxins or mutagens as further stressors to the process. Perhaps the best discussion of this is found in Conner et al (1994) NZ Journal of Horticulture 22:361-371. Perhaps the most useful direct and quantitative evidence for transformation as a mutagen is the paper of Wang et al (1996) Transgenic Res 5: 289-301 in which the authors used molecular analysis to compare the parent genotype, the T₀ offspring and a different species as an outlier. The T₀ transformants differed substantially from their parents and some of them (there was considerable variation) contained approximately half as many DNA changes as existed between the parent and the distinct species. One might reasonably argue that these T₀s were thus half way to being a distinct species. The significance of the fact that the newly introduced mutations were random changes and the distinct species consisted of variants selected by evolution, is unknown however. Nevertheless, their conclusion, reviewed in our paper and supported by others, is that plant transformation is highly mutagenic.

Wide crosses

For wide crosses, it is our view that there is too little data to pronounce on their significance one way or the other, nevertheless, much presumably depends on the safety of the donor, the amount of DNA transferred and the method used.

The concept of GRAS

Lastly, there is the question of the scientific validity of the concept of 'history of use'. Do we have the data to determine that mutagenised varieties, for example, have so far proven safe? While we do know that, in principle, diet and health are intimately connected, with respect to long term effects or effects that are at all common (eg neurological effects, allergenic effects and others), there have been no adequately controlled experiments to link individual cultivars to specific health issues and therefore we conclude that there is little useful and reliable data on this subject. (There are specifically 'healthy' cultivars like low glucosinolate oilseed rape, however these are not relevant here.)

Further Considerations

What can be said with some confidence however is that crop plants contain large numbers of distinct chemicals with diverse consequences for human physiology-both negative and positive. We also know that their expression levels can be affected by mutations and therefore introducing mutations will at some point, either cumulatively or individually, significantly alter plant metabolism to cause changes with either positive or

negative consequences for humans. In our view it is ACNFP/ACRE and EFSA's specific responsibility to ensure that such consequences are not negative. However, since we are collectively unable to distinguish the positive changes from the negative ones it therefore is appropriate to ensure that transgenic cultivars are essentially identical to their parents. Thus all changes should be treated *as if* they are negative.

This should be achievable, particularly if transgenic interventions are truly specific but it is also relevant that the transformation standard proposed in public is 'precise' and 'engineered'.

Does this set the bar unfairly high? We argue no for three reasons:

- 1) the bar is set too low for other methods of breeding;
- 2) when unanticipated phenotypes are observed in transgenics we can only guess at their molecular/biochemical origins, whereas for most other breeding methods their origins (genetic reassortment, simple mutations) are pretty clear;
- 3) it needs to be considered at the risk assessment stage that individual transgenic events are likely to be incorporated into many varieties and they are likely to be grown and consumed on an unusually wide scale. There will therefore be less protection for the public from dilution effects in the food chain.

a) The sufficiency of EFSA's guidelines

As you will be aware, Directive 2001/18 requires information on the sequences inserted or deleted at the insertion site, including their size and function. Nevertheless, we have noticed that EFSA in its assessments has typically made various assumptions about insertion sites which collectively mean that it cannot guarantee to have met the obligations of 2001/18. Some of these assumptions appear in the guidelines and some are more evident in practice:

1) Excessive reliance on southern blotting to analyse insertion sites. There is plenty of evidence that southern blotting is a blunt instrument when used to assess the presence or absence of DNA sequences present as single copies, especially in large crop genomes.

Results generated by southern analysis should be treated as hypotheses requiring confirmation by sequencing.

2) EFSA appears not to appreciate that deletions and rearrangements of plant chromosomal DNA are a common outcome of plant transformation and that when they occur they may be far more significant than the insertion itself (eg in the scientific literature the record for T-DNA insertion is loss of 13 genes and disruption of 2 others). Deletions will not be apparent from analysis of flanking DNA unless the target site (ie the wild-type sequence) is known. Without this information these mutations will almost certainly be missed and analysis of the insertion site will therefore be incomplete.

Therefore, the sequence of the target site (ie the wild-type DNA sequence) must be determined and compared with the insertion site.

3) **We recommend sequencing 50,000 base pairs either side of the transgene insertion and comparison to the wild-type DNA.** I should qualify this by defining the insertion site as the 'extent of foreign/unintended DNA insertion'. This would have two

functions 1) to identify all mutations at and linked closely to the insertion event and 2) to identify nearby genes whose expression could be altered by inserted regulatory elements.

The aim of insertion analysis

In our view, the standard should be to approve only precise insertion events which neither introduce aberrant transcripts nor affect endogenous transcripts at the insertion site. The rationale being that there is much we don't know much about the genome and precise insertion significantly reduces the possibility of unforeseen outcomes.

Phenotypic and compositional analysis

The above recommendations cover genetic, but not phenotypic consequences of plant transformation.

In principle, precise, single copy insertion into intergenic regions (assuming they exist) would obviate the need for phenotypic analysis, at least in as much as unanticipated consequences are caused by transgene insertion.

In practice, it has not so far been possible to achieve such precise insertion events and in the absence of sufficient characterisation at the insertion site, and combined with the use of highly mutagenic transformation procedures, there appears to us to be a realistic possibility that introduced mutations (even after backcrossing) will engender unanticipated consequences. EFSA's GMO panel appears to assume, and their guidelines concur, that unanticipated and unintended effects are sufficiently unlikely that they can be left to post market monitoring. We suggest that this estimation of the improbability of unanticipated consequences is a significant untested assumption. This in itself would be problematic but it is also an assumption that is contradicted by much data based not just on mutation frequency but also on phenotypic and 'omic analysis (eg Haslberger 2003 Nat. Biotech 21 739-741; Charlton et al 2004 Plant Biotech. Journal 2:27-35). With this in mind we make two specific recommendations.

1) the first stems from the fact that the consequences of mutations will be random and therefore unanticipated. This means in practice that the case-by-case approach, which relies on excluding certain hazards based on predicted function(s) of the transgene, is insufficient. **What is additionally required is that the risk from hazards identified in advance as part of the formal risk assessment process, should be addressed explicitly by experiments that are as direct, specific and realistic as possible.** Thus, for example in the case of a herbicide-resistant crop intended for human consumption, a determination of substantial equivalence does not, in our view, remove the necessity for feeding trials (to assess the hazard of human toxicity) since compositional measurements are insufficiently direct and specific to the hazard in question. **Instead, in the example of a crop destined to be fed directly to humans, specific experiments should be designed and performed to look at specific important endpoints (eg direct effects on liver health, neurological function, reproductive health, etc) in both human and animal subjects. This is the only realistic way that unanticipated consequences can be addressed effectively.**

2) that each insertion event, because it carries a unique insertion and a unique spectrum of mutations should not, at least with respect to unanticipated consequences, be considered to provide familiarity with a subsequent event.

Carrying out these recommendations would require a significant increase in the experimental content of GMO risk assessment but we believe this to be necessary and important until such time as the issues surrounding characterisation of insertion events and the mutagenic consequences of commercial transformation procedures are resolved.

I apologise for the rather lengthy answer but (without wishing to presume) I sense that there is a significant gulf between our view and that of many regulators and that the context as well as the specifics of our views are relevant in explaining our position on this complex subject.

Please contact me if we can be of further help.

yours sincerely

Jonathan Latham (PhD)

Bioscience Resource Project