

The Advisory Committee on Releases to the Environment's (ACRE's) response to concerns raised in written representations and submissions associated with the CHARDON LL public hearing and to statements made at ACRE's open hearing relating to the safety assessment of T25 GM maize conducted under Directive 90/220/EEC.

Introduction

The Advisory Committee on Releases to the Environment (ACRE) is a statutory, independent science and technical committee that advises ministers on the risks to human health and the environment from the deliberate release to the environment of genetically modified organisms (GMOs). Further information about ACRE, its remit and membership, its range of expertise and declarations of members interests, agendas, minutes, and publications are available on the ACRE website¹.

CHARDON LL² is a GM maize variety containing the T25 transformation event that confers tolerance to the herbicide glufosinate ammonium. The T25 / CHARDON LL GM maize lines were developed by AgrEvo (who subsequently became Aventis CropScience and more recently became Bayer CropScience).

In 1995, AgrEvo applied through the French authorities for approval under EU Directive 90/220 to place GM maize carrying the T25 transformation event on the European market for import as grain, cultivation and use as animal feed. In June 1995 France circulated the dossier to other member states with the recommendation that T25 maize be given approval. After due consideration, in 1998 European Union member states approved the marketing of T25 maize. As part of this process ACRE considered the French opinion and dossier supporting the application and gave its advice to the UK government in June 1996. ACRE agreed with the assessment of the French competent authority that T25 maize poses no greater risk to human health and the environment than non-GM commercial maize varieties.

¹ <http://www.defra.gov.uk/environment/acre/index.htm>

² CHARDON LL is a variety of maize containing the transformation event T25 that is approved under Directive 90/220/EEC. Transformation events are regulated under the GMO regulations; the seed legislation operates at the "variety" level.

CHARDON LL, a genetically modified (GM) fodder maize variety developed with tolerance to the herbicide glufosinate ammonium, has been proposed for addition to the National List³. Under the 1982 National List of Varieties Regulations, parties affected by the proposed decision to "list" a variety could make written representations to and/or request a hearing before a person appointed by ministers. Many of the representations received are critical of the marketing approval given for T25 maize by the European Community in 1998⁴. Further written submissions were presented for consideration when the hearing was resumed after an adjournment⁵. ACRE has considered all the relevant scientific concerns raised in written representations and submissions to the CHARDON LL hearing. In excess of fifteen box files of written material was submitted and ACRE has considered all the relevant scientific concerns expressed within it.

In addition, ACRE called an open hearing on February 20th, 2002 in which the Committee invited witnesses and independent experts to speak on scientific issues addressing four key areas associated with the risk assessment of T25 maize:

- (i) Transgene stability and the risks from horizontal gene transfer
- (ii) Environmental risk assessment and monitoring
- (iii) Compositional equivalence
- (iv) Feed safety assessment including the chicken feeding study

Witnesses included those for and against the marketing of T25 maize. The Committee's aim was to ensure that they had an accurate overview of both sides of the argument as well as having considered the relevant scientific issues. As animal feed studies were discussed, members of the

³ Before any new plant variety (GM or conventional) can be placed on the market it has to be listed on the National List of approved varieties. To be "listed" the variety has to pass minimum agronomic standards. The legislation that underpins this process and serves to protect farmers from buying sub-standard seed (the National List Regulations) is quite separate from the GMO safety legislation.

⁴ Bad Science, Bad Decisions. Friends of the Earth briefing paper, www.foe.co.uk and official proceedings of the CHARDON LL public hearing, www.defra.gov.uk/plant/pvs/CHARDON/index.htm

⁵ The CHARDON LL Hearing was adjourned in November 2000 to allow the European Commission time to clarify the lawfulness of distinctness, uniformity and stability tests carried out by the French that comprised part of the proposal to add CHARDON LL to the National List.

Advisory Committee on Animal Feedingstuffs (ACAF) augmented ACRE at the hearing.

This paper gives ACRE's advice on the concerns expressed at ACRE's hearing as well those raised in the written representations and submissions associated with the CHARDON LL hearing - it is not intended to provide a comprehensive summary of ACRE's safety assessment.

Reconsidering risk assessments in the light of new information is an important part of ACRE's remit. To this end, ACRE has continued to issue advice concerning T25 maize following its original assessment⁶. One of the features of the GM regulatory regime in Europe is that consents are not fixed and any new evidence that indicates that the product is causing harm will result in the consent being altered or withdrawn.

In the remainder of this document, points raised in written representations and submissions for the CHARDON LL public hearing and during ACRE's open hearing are in bold italicised text; these are followed by a response from ACRE.

⁶ Advice issued by ACRE in connection with T25 maize:

-Advice on notification for consent to market GM maize with tolerance to glufosinate ammonium herbicide, 20 June 1996.

- Advice for the secretary of State 23 June 1998. Genetically modified maize in national list trials adjacent to an organic farm in Devon. Available on ACRE's website:

<http://defra.gov.uk/environment/acre/pubs.htm>

- Advice for The Secretary of State 25 March 1999. A Report on The Dispersal of Maize Pollen Compiled by the National Pollen Research Unit and Commissioned by The Soil Association

ACRE's Overall Advice on Concerns Raised During the CHARDON LL Public Hearing and During ACRE's Open Hearing.

13 December 2002

ACRE has carefully considered the scientific points made in written representations and submissions for the CHARDON LL public hearing⁷ and at ACRE's open hearing that are connected with the safety of T25 maize to human health and the environment. No evidence was presented to alter ACRE's previous risk assessment of T25 maize. The Committee is therefore satisfied that its advice given to government in 1996 is valid - that there is no evidence currently available indicating that T25 maize poses a greater risk to human health or to the environment than non-GM maize varieties.

ACRE asked the UK Advisory Committee on Animal Feedingstuffs (ACAF) to advise on the feed safety assessment for T25 maize. ACAF is content that that T25 maize grain and its products pose no more risk as animal feed than non-GM maize varieties. Chemical analyses indicate that the composition of T25 maize is within the range expected for commercially grown, non-GM maize varieties. The difference is that T25 maize plants synthesise PAT protein. There is no evidence from its chemical composition, its biochemical properties nor from toxicity studies that the PAT protein confers increased allergenicity or toxicity on this GM maize line.

However, the data on the composition of T25 maize silage were limited and it was not possible for ACAF to be sure that the silage is compositionally equivalent to that of other maize varieties. Although the available data do indicate that T25 maize silage is the same as other maize and there is no reason to believe it is unsafe, ACRE agrees that its equivalence should be confirmed. Further information has been sought from Bayer CropScience⁸ (the company that hold the consent to market T25 maize) to resolve this matter.

As yet, there has been no commercial planting of T25 maize in the UK. This is not because of concerns over the safety of this GM line. One reason is that there is uncertainty about the effects that altered herbicide

⁷ The CHARDON LL hearing was instigated under the Seeds (National Lists of Varieties) Regulations 1982.

⁸ The original T25 application dossier was submitted by AgrEvo who subsequently became Aventis CropScience and more recently, Bayer CropScience.

usage associated with the widespread cultivation of T25 maize might have on farmland biodiversity. ACRE has been instrumental in drawing attention to the need to consider this issue and supports the view that the Farm Scale Evaluations are appropriately designed to tackle it. The Committee will offer scientific advice to ministers on the outcome of the evaluations once they have been completed and the results made available to ACRE. In addition, approval for the use of glufosinate ammonium herbicide on this GM maize is required and CHARDON LL must be added to the UK's National List before it can be grown commercially.

Background to the transgenic trait

At this point it is helpful to explain that the herbicidal compound L- phosphinothricin (L-PPT) to which T25 maize is tolerant, is the active ingredient in a number of herbicides with different names. Some of these have been referred to in the CHARDON LL and ACRE hearings. To avoid any confusion the names of these herbicides and the genes that confer tolerance to them are described below.

There are two species of soil bacteria known to synthesise PPT and it is the L form of this amino acid that is toxic to plants. L-PPT is the active ingredient of a number of broad spectrum herbicides, these include glufosinate ammonium and bialaphos, both of which have been referred to in association with T25 maize. Liberty® is the commercial name for the glufosinate ammonium herbicide that is linked to this crop.

Herbicides based on L-PPT act by inhibiting glutamine synthetase, an enzyme which catalyses the first reaction in the pathway that assimilates inorganic nitrogen into organic compounds. When L-PPT inhibits glutamine synthetase, toxic levels of ammonia accumulate in plant cells causing cell death. This is not the case in mammals (including humans) treated with the herbicide, since mammals have different metabolic pathways that prevent ammonia from building up and imbalances in amino acid levels from occurring⁹.

Traditional breeding programmes to generate crop plants tolerant to glufosinate ammonium have not been successful. However, using genetic modification techniques, genes from bacteria that confer tolerance to L-PPT have been transferred into a variety of plants. These genes encode enzymes that inactivate the herbicidal activity of L-PPT. The *pat* (phosphinothricin N-acetyl transferase) and *bar* (bialaphos resistance) genes both encode phosphinothricin acetyl transferases (PATs¹⁰) that catalyse the detoxification of L-PPT. In the presence of acetyl-CoA, PAT enzymes catalyse the acetylation of the free amino group of L-PPT to yield N-acetyl-L-PPT, a compound that does not inactivate glutamine synthetase. The PAT proteins that *pat* and *bar* genes encode are considered to be functionally equivalent for the purpose of conferring tolerance to L-PPT (Wehrmann et al 1996¹¹). Both forms of the enzyme are highly specific for L-PPT and do not acetylate other L-amino acids.

T25 maize contains the *pat* gene from a laboratory strain of a naturally occurring soil bacterium, *Streptomyces viridochromogenes*¹², Tu494 (Wohlleben *et al.* 1988¹³).

⁹ Hack R., Ebert E., Ehling G. and Leist K-H. (1994). Glufosinate ammonium – some aspects of its mode of action in mammals. *Food Chem. Toxicol.* **32**: 461 –470.

¹⁰ The PPT acetyltransferase encoded by the *bar* gene is sometimes referred to as a BAR, rather than a PAT enzyme.

¹¹ Wehrmann A., Van Vliet A., Opsomer C., Botterman J. and Schulz A. (1996). The similarities of *bar* and *pat* gene products make them equally applicable for plant engineers. *Nature Biotech.* **14**: 1274 – 1278.

¹² This species of *Streptomyces* is a saprophytic, soil-borne microbe and is not considered a pathogen of plants, humans, or other animals.

Environmental Issues

Point 1. *It has been asserted at the hearings that ACRE did not consider the effects on soil ecology resulting from the horizontal gene transfer of herbicide tolerance and antibiotic resistance transgenes from CHARDON LL to soil bacteria.*

Part of ACRE's risk assessment for any GMO being considered for release into the environment is its potential impact on the soil ecosystem. For T25 maize, this includes the likelihood of transgenic DNA from this GM plant line (especially from its detritus) being taken up by soil bacteria and the consequences of this for soil ecology.

In the laboratory, conditions can be applied to force horizontal gene transfer between plant and bacterial cells, albeit at a very low frequency. However, there is no convincing evidence that horizontal gene transfer between plants and bacteria takes place in the soil under natural conditions. Two published studies designed to measure horizontal gene transfer between plants and microbes in the soil have been quoted as demonstrating that this phenomenon occurs¹⁴. However, the authors of these publications did not demonstrate, nor did they claim to provide evidence of, horizontal gene transfer between plants and bacteria under laboratory conditions or in field studies (see Point 2 for more details). This is not to say that that horizontal gene transfer between plants and bacteria could not happen, but the evidence suggests that if it does, it is a very rare event.

Taking a precautionary approach, ACRE assumes that horizontal gene transfer could occur between T25 maize and soil bacteria and then considers the implications. Horizontal gene transfer can only impact on soil microflora if the *pat* gene is expressed following transfer and if this expression confers a selective advantage to the recipient bacterium.

The cauliflower mosaic virus (CaMV) 35S promoter drives *pat* gene expression in T25 maize. If this promoter was transferred into a bacterial genome ACRE's view is that its capacity to regulate gene expression would be negligible. ACRE accepts there may be very low levels of activity, but would emphasise that the expression of genes regulated by the CaMV 35S promoter in bacteria is minimal.

The amount of PAT protein that could potentially be synthesised is also affected by the ability of a bacterium to translate any *pat* gene expression into protein. The *pat* gene in T25 maize has been modified to optimise the synthesis of the PAT protein in plants and to minimise its translation in bacteria. To achieve efficient expression of bacterial genes in plants it is common for researchers to modify the DNA codon usage

¹³ Wohlleben W., Arnold W., Broer I., Hillemann D., Strauch E. and Puhler A. (1988). Nucleotide sequence of the phosphinothricin N-acetyltransferase from *Streptomyces viridochromogenes* Tu494 and its expression in *Nicotiana tabacum*. *Gene* **70**: 25-37.

¹⁴ Frank Gebhard & Kornelia Smalla (1998). Transformation of *Acinetobacter* sp. Strain BD413 by transgenic sugar beet DNA. *Applied and Environmental Microbiology* **64** (4) 1550-1554.

Frank Gebhard & Kornelia Smalla (1999). Monitoring field releases of genetically modified sugar beets for persistence of transgenic plant DNA and horizontal gene transfer. *FEMS Microbiology Ecology* **28**, 261-272.

pattern by altering the ratio of C/G base pairs to A/T base pairs. This does not result in an alteration to the sequence of amino acids that make up the final protein. As the *pat* gene isolated from *Streptomyces* spp. has a relatively high content of G/C base pairs when compared to plant genes, the ratio of base pairs was modified prior to introduction into maize. As a result, the modified gene is expected to lead to significantly less PAT protein in the bacterium that acquires it compared to the naturally occurring form of this gene.

ACRE also assessed the possible consequences to soil microflora if horizontal transfer of the transgene did occur and if it was then translated into an active protein. An important consideration in the risk assessment is whether L-PPT resistance is a trait that already exists in the UK's soil microbes. The fact that the particular L-PPT resistance gene used to transform T25 maize is specific to a laboratory strain of *Streptomyces viridochromogenes* (Wohlleben *et al.* 1988¹⁵) not present in UK soil bacteria was raised at the ACRE hearing. However, there are species of *Streptomyces* common to UK soils that do have L-PPT resistance genes. These include the *bar* genes of *Streptomyces hygroscopicus* (Thompson *et al.* 1987¹⁶) and *Streptomyces coelicolor* (Bedford *et al.* 1991¹⁷). Results from preliminary studies carried out by Dr. Jeremy Sweet at NIAB were quoted at ACRE's hearing as evidence for the lack of existing L-PPT resistance genes in UK soils. We understand from Dr. Sweet that he believed that his failure to amplify *bar* gene DNA to detectable levels was due to problems with the method used and could not be attributed to a lack of *bar* gene DNA in his soil samples.

Having established that L-PPT resistance genes exist in UK soil microbes, ACRE then sought evidence for *bar/ pat* gene expression in UK soil or its counterparts. There are relatively few studies on the occurrence of PAT activity in soil bacteria. However, Bartsch and Tebbe (1989)¹⁸ showed that of the 300 diverse bacteria isolated from German soil samples, 2% exhibited PAT activity. Species of bacteria shown to have PAT activity in this research are also common in UK soils (*eg. Rhodococcus, Alcaligenes, Agrobacterium, Serratia* and *Pseudomonas*). In addition, this study identified enzyme activities that catalyse the degradation of the herbicide in a different way from the PAT protein.

One of the characteristics that led AgrEvo to select the T25 maize line for commercialisation was that it does not have an intact ampicillin resistance gene¹⁹.

¹⁵ Wohlleben W., Arnold W., Broer I., Hillemann D., Strauch E. and Puhler A. (1988). Nucleotide sequence of the phosphinothricin N-acetyltransferase from *Streptomyces viridochromogenes* Tu494 and its expression in *Nicotiana tabacum*. *Gene* **70**: 25-37.

¹⁶ Thompson C. J., Movva N.R., Tizard R., Crameri R., Davies J.E., Lauwereys M. and Botterman J.(1987). Characterisation of the herbicide resistance gene *bar* from *Streptomyces hygroscopicus*. *EMBO J.* **6**: 2519-2523.

¹⁷ Bedford D. J., Lewis C.G. and Buttner M.J. (1991). Characterisation of a gene conferring bialaphos resistance in *Streptomyces coelicolor*A3(2). *Gene* **104**: 39-45.

¹⁸ Bartsch K. and Tebbe C.C. (1989). Initial steps in the degradation of phosphinothricin (glufosinate) by soil bacteria. *Applied and Environmental Microbiology* **55** (3): 711-716.

¹⁹ Northern blots and enzyme assays indicate that the gene is not transcribed and that no antibiotic activity is evident in the maize tissue.

Therefore horizontal gene transfer of antibiotic resistance is not an issue with T25 maize.

Taking all the evidence summarised above, the Committee's view is that the likelihood of genes moving from plant debris to soil bacteria is very low and that the environmental consequences (if it did occur) with this particular GM maize would not be significant. It is noteworthy that genes conferring PAT tolerance are already present in soil bacteria and it is much more likely that they will be transferred between bacteria by horizontal gene transfer than between plants and bacteria. Horizontal gene transfer is a well documented mechanism by which bacteria exchange genetic material in the environment.

Point 2. *There has been concern that research by Gebhard and Smalla (1998 and 1999)²⁰ shows that horizontal gene transfer can take place between plants and bacteria in the soil.*

In July 2000 ACRE reviewed these papers by Gebhard and Smalla (1998 and 1999) and concluded that they provide important information and confirm advice given previously²¹. The authors of these publications did not demonstrate, nor did they claim to provide evidence of horizontal gene transfer under laboratory conditions or in field studies. Horizontal gene transfer between plant and bacterial cells can be achieved by using forced conditions in the laboratory and by supplying pressure to select for the gene product and consequently the transgene itself. Although there is no evidence that this process actually exists under natural conditions it remains a theoretical possibility. Therefore, taking a precautionary approach, ACRE's safety assessments assume that it might occur. (see Point 1 above).

Point 3. *Concern has been expressed that ACRE did not modify its advice in response to a report by the National Pollen Research Unit²².*

²⁰ Frank Gebhard & Kornelia Smalla (1998). Transformation of *Acinetobacter* sp. Strain BD413 by transgenic sugar beet DNA. *Applied and Environmental Microbiology* 64 (4) 1550-1554.

Frank Gebhard & Kornelia Smalla (1999). Monitoring field releases of genetically modified sugar beets for persistence of transgenic plant DNA and horizontal gene transfer. *FEMS Microbiology Ecology* 28, 261-272.

²¹ ACRE Annual Report Number 7 (2000) ANNEX H. Advice to the Secretary of State on Horizontal gene transfer: genetically modified crops and soil bacteria. Available online: <http://www.defra.gov.uk/environment/acre/index.htm>.

²² Emberlin J., Adams-Groom, B. Tidmarsh, J. (1999) A Report on the Dispersal of Maize Pollen. National Pollen Research Unit, University College, Worcester. Report commissioned by and available from the Soil Association, Bristol House, 40-56 Victoria Street, Bristol, BS1 6BY

In 1999, ACRE reviewed the report published by The National Pollen Research Unit on maize pollen dispersal to determine if it raised any evidence not available when ACRE advised, in 1998, on the cross-pollination between an organic sweetcorn crop and a GM fodder maize in National List trials at a site in Devon²³. Specifically, ACRE considered whether its previous advice needed updating in the light of this National Pollen Research Unit report²⁴.

The Committee agreed with the main conclusions of the report *i.e.* that maize pollen can be carried by wind and insects over long distances, but found that the report did not provide any new information on cross pollination/ hybridisation frequencies. The report from the National Pollen Research Unit considered pollen as particles and did not take into account viability. Most pollen from *Poaceae* (the grass family of which maize is a member) has a short viability, which restricts the opportunities for gene flow. ACRE uses information based on hybridisation frequencies as this provides a much more accurate prediction of gene flow. These data derive from years of international experience growing conventional crops for seed production as well as data from a wealth of scientific studies measuring gene flow.

With respect to the GM maize grown in Devon in 1998, ACRE advised specifically on this case and took into account local topography, size and number of plots (pollen dilution was a key issue as only 6 plots out of approximately 1800 were GM at the Dartington site). It also took into consideration cross-pollination rates for maize, which is an internationally recognised criterion for ensuring high purity in seed production (UK Seeds Regulations, EC Seeds Directive and OECD Maize Seed Scheme). At a separation distance of 200 metres the Committee concluded that cross-pollination events between the organic crop and its GM counterpart would account for no more than 1 hybrid sweetcorn kernel in every 40,000.

In contrast, the National Pollen Research Unit's report on pollen dispersal proposed that 1 kernel in 93 could be a hybrid at a separation distance of 200 metres. The National Pollen Research Unit's calculation of hybridisation frequencies is an approximation based on mathematical modelling. The report stated as much and qualified the estimate by stating that a whole variety of environmental factors could vary the accuracy of this estimate such as local topography, field size etc.

Whilst ACRE considers modelling experiments on pollen flow interesting and useful, data from hybridisation studies used in conjunction with local details are much more accurate in predicating gene flow. ACRE therefore concludes that the National Pollen Research Unit's report on pollen dispersal does not alter its previous advice.

²³ Advice for the secretary of State 23 June 1998. Genetically modified maize in national list trials adjacent to an organic farm in Devon. Available on ACRE's website:
<http://defra.gov.uk/environment/acre/pubs.htm>

²⁴ Advice for The Secretary of State 25 March 1999. A Report on The Dispersal of Maize Pollen Compiled by the National Pollen Research Unit and Commissioned by The Soil Association

A number of other reports associated with gene flow from maize have been submitted to the CHARDON LL hearing. ACRE is aware of these and the local and seasonal variation that their results imply and it is content that the separation distances employed at the Dartington trial site minimised the probability of T25 maize hybridising with non-GM lines. It is important to note that separation distances are pragmatic – they are based on years of international experience producing high purity seed as well as from numerous scientific studies measuring hybridisation frequencies. They do not and were never intended to provide complete genetic isolation and as such GM crop varieties are only released into the environment if the consequences of gene flow are assessed as not presenting a greater risk to the environment or human health compared to their non-GM counterparts.

Point 4. *Concern has been expressed that ACRE did not evaluate the safety of CHARDON LL in accordance with the more stringent amendments to Directive 90/220/EEC and the EU environment Minister's declaration of December 1998.*

Until December 1998, the EU competent authorities had not agreed a harmonised approach to risk assessment. It was therefore important that some clear principles reflecting best practice in EU member states were formalised. In 1998, negotiations to amend the existing directive, 90/220/EEC started. In October 2002, Directive 90/220/EU was repealed and replaced by Directive 2001/18/EU. This new directive contains a number of procedural changes and amongst these are two new annexes, one concerns the principles for risk assessment (Directive 2001/18/EU: Annex 2) and another on post-market monitoring (Directive 2001/18/EU: Annex 7).

The new procedures reflect the issues that member states have considered during their safety assessments since Directive 90/220/EEC came into force in 1992. A fundamental property of the directive is that each member state makes an independent assessment of every application submitted to place a GMO on the market and these views are then shared before a final decision is made. A diversity of views and concerns are therefore considered for each application before member states collectively decide whether or not to grant a marketing consent.

With respect to Annex II of the new directive and its reference to direct, indirect, immediate and delayed effects – ACRE has always considered these aspects in risk assessments and this is evident from its minutes and advice. For example, ACRE's consideration of non-target/indirect effects includes any possible consequences resulting from the insertion of transgenic DNA into a host's genome. The transfer of transgenes to wild relatives and any environmental consequences that result, are a further example of ACRE's consideration of indirect effects. This is also an example of a delayed effect since environmental impact must be viewed over several generations.

It was possible under the original directive (90/220/EU) for member states to agree to impose post market monitoring. For maize varieties such as CHARDON LL, ACRE was satisfied that there was no evidence to suggest they would behave any differently from conventional varieties. On this basis, ACRE was confident that the maize did not need to be monitored over and above the normal quality control and inspection tests

that are observed for all plant varieties. When the decision to issue a consent to place T25 maize on the market place was made in April 1998, no member state called for post-market monitoring.

The UK Farm Scale Evaluation (FSE) programme²⁵, designed to assess the potential impact of farm management practices associated with the cultivation of herbicide-tolerant GM crops, has not yet been completed. No commercial planting of T25 maize will take place until the FSEs have concluded in 2003 and only then, if the use of L-PPT herbicides on maize is considered to cause no unacceptable effects on the environment. Approvals for the use of the herbicide on maize under pesticide regulations and the addition of T25 maize to the National Seed List are also required before it can be grown commercially in the UK.

ACRE's risk assessment procedures have been in line with the new directive for a number of years; indeed, UK Government scientists and ACRE have been pivotal in guiding the revisions for products considered at EU level under part C applications.

Point 5. *It has been asserted that ACRE did not consider the possibility of genetic instability in T25 maize.*

ACRE is satisfied that the transgenic material is inserted stably and there is no evidence that T25 maize is any less stable than its conventionally bred, non-GM counterparts. This assessment includes a consideration of transposable element activity²⁶ and changes in the methylation state of DNA²⁷. The original T25 maize line was created in the late 1980s. It was selected from a large number of transformed lines based on its molecular characteristics *i.e.* it has a single, relatively short insert with an incomplete ampicillin resistance gene. Subsequently, the transgene has been expressed under different genetic and environmental conditions through numerous generations without any evidence of instability²⁸; in each case the *pat* gene conferred the expected tolerance to the herbicide.

During ACRE's hearing it was implied that T25 maize had failed the original distinctness, uniformity and stability (DUS) tests (criteria on which proposals to add varieties to the National List are partially based) on safety grounds - this is not the case⁵. There is also no evidence in the literature to support the idea that transgenic DNA is inherently less stable than native DNA. It was pointed out by one of the expert witness at ACRE's hearing that all genomes are unstable to a certain degree and that there is no particular reason why transgenic DNA should be less stable unless it carries with it mechanisms for insertion and excision. As previously discussed there

²⁵ Detailed papers on the history and science of the Farm Scale Evaluations are available: <http://www.defra.gov.uk/environment/fse/index.htm>

²⁶ Transposable elements occur naturally in many plants, including maize; they can move at low frequency from one genetic location to another

²⁷ The methylation status of DNA can affect the expression of genes

²⁸ Bayer CropScience estimate 23 generations of breeding, with 40 different maize varieties world-wide now containing the T25 insert.

is no evidence in successive generations of T25 maize lines that the inserted sequence and therefore any genetic elements contained within it, confer instability.

Point 6a. *It has been suggested that the possibility of T25 maize being toxic to bees has not been considered.*

The application dossier submitted by AgrEvo in 1995, did consider the possibility that T25 maize could be toxic to beneficial insects. ACRE assessed the impact that T25 maize might have on beneficial insects such as bees taking into account their exposure to the transgenic product and consequently what effect the PAT protein might have. It is important to note that the genes themselves are not toxic; it is only the gene products (*i.e.* proteins) that have the potential to be toxic. Therefore, one consideration is whether the transgene is translated into protein (*i.e.* expressed) in particular tissues or structures, such as pollen.

T25 maize has been grown commercially as well as in research and development trials in Europe and the United States for many years and there is no evidence that it causes harm to bees.

ACRE considers that T25 maize poses a negligible risk to bees since:

- There is no evidence that PAT is toxic to bees.
- Tests show that PAT enzymic activity is very specific for its substrate, PPT and it is only species within the genera *Streptomyces* and *Kitasatosporia* that have been reported to synthesise this amino acid.
- Analyses show that PAT protein and enzymatic activity is absent in CHARDON LL pollen

Point 6b. *Concern has been expressed that bees could be at risk from horizontal gene transfer of the pat gene.*

It has been suggested from preliminary studies by Dr Hans-Hinrich Kaatz²⁹ that the *pat* gene from T25 maize could be transferred to bacteria in the intestinal tracts of bees exposed to its pollen. Since this work has not been published, ACRE cannot give its view. However, in line with the precautionary principle, ACRE does consider the possibility of horizontal gene transfer of transgenes to microorganisms and there is no evidence in this case, that it would have an adverse effect.

Point 7. *There has been some concern that dust and sap from T25 maize plants might adversely affect humans and bees respectively.*

Chemical analyses demonstrate that T25 maize is compositionally equivalent (see Point 12) to its non-GM counterparts except for the presence of PAT protein. All the available evidence supports the conclusion that this protein does not confer increased toxicity (*e.g.* toxicity studies and biochemical properties) or allergenicity (the PAT protein does not have significant homology to any known allergen) on T25 maize

²⁹ University of Jena and the Hans Knöll Institute for Natural Substance Research in Jena.

compared to non-GM maize varieties. Therefore, there is no evidence for, or reason to expect that sap, crop dust or any material derived from T25 maize plants presents an increased risk to human health or the environment compared to matter from conventional maize varieties.

Point 8. *It has been asserted that the possible production of unidentified biochemical products in maize crops and wild relatives resulting from cross pollination with CHARDON LL was not considered.*

As there are no sexually compatible wild relatives of maize in Europe, transfer of genes *via* cross-pollination events can only occur between CHARDON LL and other maize varieties. If CHARDON LL did hybridise with a different maize line the transgenic DNA would still be in a very similar genetic background. Since the transgene does not result in the production of unexpected toxins or allergens in CHARDON LL there is no reason for this to be any different in a hybrid. In addition, there is no evidence from a detailed knowledge of the mode of action of PAT that it would alter the metabolism of maize hybrids any differently from CHARDON LL.

The PAT enzyme has been well-characterised biochemically³⁰; it is highly specific for its substrate, L-PPT (an unusual amino acid) and is incapable of catalysing reactions involving other closely related compounds (including other amino acids). This indicates that PAT is very unlikely to interfere with other plant processes.

Point 9. *There has been a suggestion that the cauliflower mosaic virus promoter is unsafe and that its use can lead to gene silencing and DNA rearrangements.*

The expression of the *pat* gene in T25 maize is regulated by the cauliflower mosaic virus (CaMV) 35S promoter. ACRE has considered the CaMV 35S promoter on a number of occasions and in particular reviewed the paper by Ho *et al.*, (1999)³¹. The Ho *et al.* paper discusses the potential for the CaMV 35S promoter to recombine with other virus DNA and the role that this promoter might play in horizontal gene transfer. The paper reviews the scientific literature on the CaMV 35S promoter and advances a hypothesis that a 'recombination hotspot' predisposes the promoter to recombine with virus, plant and animal DNA that has a similar nucleotide sequence. Ho *et al.* suggest that this may lead to the generation of new pathogenic viruses or to the unexpected and harmful over-expression of plant genes located in close proximity to the site of recombination. It was also suggested that recombination events with animal DNA might even lead to cancer. However, no new data or direct experimental

³⁰ In the presence of acetyl-CoA as a co-substrate, PAT catalyses the acetylation of the free amino group of L-PPT to yield N-acetyl-L-PPT, a compound that does not inactivate glutamine synthetase. Both of the PAT enzymes are highly specific for L-PPT and do not acetylate other L-amino acids, nor do they acetylate D-PPT. In the presence of excess concentrations of L-amino acids, both PATs also are unaffected in their ability to acetylate L-PPT. In L-PPT tolerant plants which express relatively high levels of PAT, the main residue metabolite of L-PPT catabolism is N-acetyl-phosphinothricin

³¹ Ho, M. W., Ryan, A., Cummins, J.(1999). Cauliflower mosaic viral promoter – a recipe for disaster. *Microbial Ecology in Health and Disease* **11** (4)

evidence is presented to support the authors' hypothesis that the CaMV 35S promoter, used in many GM plants, is inherently dangerous.

For many thousands of years CaMV and its relatives have infected plants; consequently humans and animals have been eating plant material containing the 35S promoter *via* natural CaMV infection. No ill effects due to the activity or recombination of the virus promoter have been reported and in particular, no reports of cancer. In fact, Brassica crops such as broccoli, which usually carry at least some CaMV infection, are implicated in protection from cancer.

Ho *et al.*, speculate that the 35S promoter has a tendency to recombine with other DNA which could have harmful consequences. However, there is no evidence for this, or any reason why 35S CaMV DNA would be more prone to recombination than other DNA that does not have elements associated with insertion and excision.

A prominent concern raised in this paper is that the 35S promoter in GM plants could inadvertently activate dormant viruses or non-target genes in plants or other organisms. ACRE is aware that the 35S promoter has the potential to alter the expression of host genes neighbouring the site of its insertion. This is one of the reasons why ACRE require applications for marketing consents to describe the host DNA that flanks the site into which the transgene has inserted. In addition, phenotypic and compositional studies are used to determine whether the direct or indirect effects of an insertion event alter important characteristics of the transgenic line. There are no reported incidences of a dormant plant virus being unintentionally activated by the insertion of a transgene with a 35S promoter. In particular, there is no evidence that the T25 insertion event has altered the maize line in any way that makes it less safe to human health or the environment than its conventional counterparts.

Ho *et al.*, have suggested that there is a '*close relationship*' between CaMV and human viruses such as hepatitis B and that CaMV 35S promoter DNA inserted into transgenic plants will recombine with DNA from these viruses. However, CaMV and human retroviruses are not members of the same genetic family and the degree of similarity between their DNA sequences is low. It is also worth considering that if recombination between plant and animal viruses, perhaps in the gastrointestinal tract, were a realistic possibility then the abundance of plant virus DNA in the diet would mean that this was a frequent natural occurrence and it is not.

In conclusion, the paper by Ho *et al.*, does not challenge current scientific understanding or indicate that the CaMV 35S promoter, as used in genetic modification, is inherently unsafe. The CaMV 35S promoter used in this way represents an extremely low risk to human health and the environment.

Animal Feed Safety Issues

Point 10. *It has been suggested at the CHARDON LL hearing that the Advisory Committee on Animal Feedingstuffs (ACAF) has not considered the safety of T25 maize as animal feed.*

At the time that the T25 maize application dossier was under review in 1996, ACAF did not exist. At that time ACRE was not constituted to advise in detail on feed safety and so the dossier (like others) was referred to animal feed experts at the Ministry of Agriculture, Fisheries and Food (MAFF). In addition, the Advisory Committee on Novel Foods and Processes was asked for its advice on the safety of T25 for use as food and in animal feed - it was content that the risks were very low. In the absence of objections expressed to ACRE by MAFF and particularly in the light of the ACNFP's views, ACRE was content for a favourable opinion to be forwarded to the European Commission. It is noteworthy that the dossier had been considered in detail by the authorities in France where it was originally submitted and subsequently it has been assessed by other member states and by the commission's Scientific Committee for Plants.

ACAF was established in June 1999 specifically to provide government with independent expert advice on all issues relating to the safety and use of animal feedingstuffs. So in 2000, when ACRE was asked to review T25 maize in the light of views expressed at the CHARDON LL hearing, it asked ACAF for advice on animal feed aspects. ACAF is satisfied that on the basis of the information available, there is nothing to indicate that T25 maize grain or its products pose any more risk to humans or animals than non-GM varieties if used in animal feed.

Point 11. *It has been asserted that the 14 day duration of the rat toxicity test does not realistically model toxicity caused over lifelong exposure.*

AgrEvo commissioned a repeated dose, oral toxicity test in rats to assess the toxicity of the PAT protein. The body weights, organ weight and food consumption of rats were measured. There were no statistically significant differences in these parameters in rats fed PAT protein compared to rats in control groups. The duration of this study has been criticised as being too short; but this is a recognised procedure whereby test animals are fed 'large' amounts of a specific substance over a relatively short period and monitored for any toxic effects. After two weeks of this regime, there was no apparent effect on the animals indicating that PAT is not acutely toxic.

In ACAF's view, the rat study was not entirely without criticism. Members considered that the origin of the PAT protein fed to rats was obscure and the use of activity units and weight of PAT did not allow the level of exposure to be determined accurately. On request the company clarified these points: PAT protein was synthesised in bacteria (*Escherichia coli*) and the dosage fed to rats in this study was at least 1000 times greater than dietary exposure from eating GM plants.

There has been some criticism in written representations and submissions that the PAT protein used in the rat toxicity study was synthesised in a bacterium – the

suggestion being that PAT protein made in *E. coli* may undergo post-translational modification causing differences in stability compared to PAT synthesised in plants. However, PAT protein from both sources was passed through a matrix along an electric gradient (gel electrophoresis) and no differences in their respective mobilities were observed. This suggests that no significant post-translational modification of the PAT protein synthesised in the bacterium has occurred. The use of bacteria to generate large amounts of pure recombinant protein is normal practice because the alternative generally requires the production of considerable quantities of plant material.

Point 12. *It has been suggested that the chicken feeding study was a poorly designed experiment and that a trend for higher mortality in GM fed broiler chickens gives cause for concern. The nature of the trial has also been criticised i.e. the suitability of feeding T25 maize grain to chickens when the target organism are ruminants that will be fed T25 maize silage.*

The chicken feeding study was not part of the application dossier submitted to the French competent authority by AgrEvo in 1995 – it was submitted afterwards to support conclusions on the nutritional equivalence of T25 maize grain with its non-GM counterparts. The UK government did not ask ACRE to assess a report of the chicken feeding study that was circulated to member states by the French in 1997. The chicken feeding study was not considered by ACRE until 2001 when it asked for ACAF's advice on animal feed aspects of the T25 maize dossier in response to criticism raised at the CHARDON LL hearing.

In the original T25 maize dossier submitted by AgrEvo in 1995, conclusions on nutritional equivalence were based on the results of compositional analysis; this is in accordance with accepted practice. ACRE was satisfied that the T25 maize grain is compositionally equivalent to conventional maize grain except for the presence of the PAT protein and that this protein poses a negligible risk to animal or human health.

ACRE accept that broiler chickens are a sensitive test system but are not convinced of their relevance in assessing the 'wholesomeness' of T25 maize since ruminants are the target organism for this product and these will be fed T25 maize silage, not grain. At ACRE's hearing, it was suggested that a laboratory-based study using microbes found in the guts of ruminants would have been more informative than a chicken feeding study. ACRE is concerned that applicants should consider the relevance of any feeding trial that is carried out and its limitations.

However, as the chicken feeding study was submitted for consideration, ACRE is clear that, it should meet the standards expected for information presented in application dossiers. This means that experimental design, including statistical analyses should be sufficient to address questions asked of the study and clear, detailed descriptions of both should be supplied.

The chicken feeding study was conducted in 1996. In 1997, the French circulated a report of the study to other member states. In the UK, MAFF were asked to lead on this (ACAF had not been established at this point)- the report was not sent to ACRE as it was not constituted to advise in detail on animal feed safety. MAFF did not

report any objections and the UK agreed with the French that the chicken feeding study did not alter the risk assessment of T25 maize.

During the CHARDON LL public hearing and subsequently at ACRE's open hearing, the relevance of the chicken feeding study, its experimental design and statistical analysis were criticised. Concern was also expressed about mortality rates in birds fed T25 maize grain compared to those fed grain from a non-GM variety. In the light of these concerns, ACRE asked ACAF for its advice on the safety of T25 maize for use in animal feed.

The chicken feeding study was carried out at the Department of Animal and Poultry Sciences at the University of Guelph in Canada. Broiler chickens were chosen as the target species because they provide a very sensitive test system. During the first 18 days of life the body weight of broiler chicks increases 15-fold, therefore inadequacies in diet are quickly apparent. The study took place over forty two days and involved 280 male birds, half were fed T25 maize grain and the other half, non-transgenic grain.

An expected mortality rate of 5-8% was quoted for male broilers kept at the research facility at the time of the study. Therefore, under the conditions used in this experiment the expectation was that up to 11 birds would die in each group of 140 birds (8%).

The mortality rate of chickens fed T25 maize grain was within the expected range for chickens reared at the facility (10 dead out of 140 birds, *i.e.* 7%), whereas the mortality rate of chickens fed non-GM maize was lower than predicted (5 dead out of 140 birds, *i.e.* 3.6%). Concerns over the mortality rate of birds fed T25 maize have been raised; however, these are within the expected range for chickens reared under the conditions used in this study.

The power of the study to resolve differences in growth rate, if they exist, between chickens fed T25 maize grain and those in the control group is limited. There are opposing views on whether further statistical analysis of the data could increase the power of this study to answer questions on the nutritional equivalence of T25 maize. The differing points of view expressed by statisticians at the hearings are:

(i) If analysed differently, the sensitivity of this study to detect any small difference in the growth rates of T25 maize fed birds and those fed non-GM grain might be increased.

(ii) Flaws in the experimental design of the chicken feeding study would prevent any conclusions from being made, irrespective of whether the data is further analysed or not. The flaws identified were - insufficient replication of treatments (GM and non-GM fed birds) and the lack of a positive control treatment that could demonstrate whether the study has the power to detect a known decrease in growth rate. The study was also criticised for being inadequately described *i.e.* the detail given was not sufficient to repeat the experiment.

ACRE has asked the company to determine whether reanalysis of the raw data from the chicken feeding study could increase the power of this experiment in comparing

the nutritional value of T25 maize grain with that of a non-GM counterpart. This has been done and ACRE is awaiting the company's formal response.

In summary, the chicken feeding study was designed to consider unexpected, undefined characteristics of T25 maize grain that could impact on its nutritional status. There is no evidence from this study that T25 maize grain fed to animals would pose an increased risk compared with conventional maize grain. ACRE has reservations about the usefulness of this study in the risk assessment of T25 maize as animal feed. ACRE welcomes as much relevant information in application dossiers as possible, however data submitted must meet expected standards as previously described. To this end, ACRE has asked that the company determine what potential this study has to compare the nutritional status of T25 maize grain with its non-GM counterparts in chickens.

ACAF is satisfied that on the basis of the information available, there is nothing to indicate that T25 maize grain or its products pose any more risk to humans or animals than non-GM varieties if used in animal feed³³.

The company have commissioned a dairy cattle feeding study with T25 maize and will present the data to the French competent authorities when completed. As is standard practice, ACRE will review new information generated in this trial and update the risk assessment accordingly.

Point 13. *It has been asserted that composition analyses have shown statistically significant differences between T25 and non-GM maize varieties.*

While the principle of substantial equivalence has received wide international support it has also been subject to criticism. In some cases there appears to be a mistaken perception of how substantial equivalence is applied. It is not a safety assessment in its self; substantial equivalence is a starting point for identifying differences between a transgenic variety and an appropriate comparator that has a history of safe use. The equivalence of a transgenic variety's chemical composition, including a range of nutritionally important parameters and any naturally occurring toxins, is compared to its conventional counterpart. The next step would then be to determine the relevance of any differences that are found; this may involve further detailed tests. Substantial equivalence is therefore a starting point in risk assessments and it is only one part of a safety assessment. Other aspects may include toxicology experiments, comparisons between the amino acid sequence of transgenic proteins and that of known allergens and characterisation of transgene insertion sites in host genomes.

The use of compositional analysis is considered an indicator of nutritional status. At ACRE's hearing it was suggested that additional feeding studies are necessary to establish nutritional equivalence between T25 maize and its non-GM counterparts – this is discussed in point 14.

The Compositional Equivalence of T25 Maize Grain

³³ ACAF's advice on the safety of T25 maize for use in animal feed was issued to ACRE on 5 September 2001.

Compositional data for T25 maize grain are within the normal expected range for commercially grown maize varieties. This supports the view that T25 maize grain is compositionally equivalent to these varieties apart from its synthesis of PAT protein. A detailed risk assessment of PAT was conducted separately and includes assessments of potential toxicity and allergenicity. ACRE is satisfied that any risk posed by the PAT protein to animal or human health is negligible.

Some statistically significant differences were observed between T25 maize grain and its non-GM counterpart but these values are within the accepted values for conventional maize lines. Variation in the chemical composition of maize principally arises because it is a hybrid and breeders intentionally use parental lines with as much genetic distance as possible. In addition, agronomic and environmental conditions also affect composition. Because of this, animal feed experts in ACAF felt that differences between T25 maize and its non-GM counterpart were unlikely to be significant because livestock already experience subtle differences in the composition of maize.

There has been some disagreement as to the compositional equivalence of T25 maize grain based on data submitted to the CHARDON LL hearing. This centred on whether linolenic and arachidic fatty acid contents in T25 maize grain were within the range expected for non-GM maize varieties grown commercially. ACAF are content that T25 maize is compositionally equivalent to non-GM maize. Further analyses conducted since the original composition data was submitted support this³⁴.

The relevance of chemical analyses on maize grain for establishing nutritional equivalence has been questioned since it is silage that will be fed to ruminants.

The Compositional Equivalence of T25 Maize Silage

ACAF experts considered that current data for T25 maize silage is not sufficient for conclusions about equivalence to be made, however, the data presented give no cause for concern. ACRE agrees with ACAF's view that sufficient additional analytical data for T25 maize silage should be provided to demonstrate whether it is substantially equivalent to silage from conventional maize varieties. Alternatively data from a feeding study made with dairy cattle could be used to demonstrate the wholesomeness of the silage and a lack of any effect on milk production and composition. Milk production data is usually taken as a relatively sensitive indicator of body condition.

Bayer CropScience will submit further compositional data for both grain and silage and this will be reviewed as part of a continuing risk assessment.

The company also propose to submit the details of a feeding study in dairy cattle that will be used to examine the wholesomeness of T25 maize silage and any effect on milk production and composition.

³⁴ For example, please refer to data from Flachowsky *et al.* 2000 submitted to ACRE's open hearing by Professor Phipps: http://defra.gov.uk/environment/acre/hearing200202/pdf/t25_phipps.pdf. This data has been accepted for publication in a peer-reviewed journal.

Point 14. *Concern has been expressed that no tests were carried out to check that T25 maize is safe for livestock*

The fact that no feeding studies have been carried out with T25 maize in cattle or sheep has been claimed to represent a weakness in the risk assessment process. ACAF has made its position of animal feed trials clear – it recognises the value of animal feeding studies on target species, particularly for determining nutritional adequacy but does not require that animal tests are carried out in all cases. Instead, ACAF promote the use of appropriate techniques that provide the detail and quality of information required by members to assess feed safety.

ACAF is not of the view that feeding studies with T25 silage must be done. Members considered that if compositional and agronomic equivalence can be demonstrated and if a detailed risk assessment of the transgenic protein provides no indication that it is harmful to livestock then feeding studies are not essential. In the case of T25 maize grain, chemical analyses have demonstrated compositional equivalence with non-GM, commercially available counterparts and there is no evidence from a variety of studies that the PAT protein poses a risk to livestock (*i.e.* its biochemical characteristics, its fate in gastric juices and the lack of similarity to any known toxin or allergen). However, further data are required to establish whether T25 maize silage is substantially equivalent to non-GM varieties grown commercially (refer to Point 12).

The use of compositional studies to assess the nutritional value of T25 maize to ruminant animals has been criticised. The point made was that chemical analyses, including measurements of fibre content, are a poor predictor of the digestibility of plant matter and that lowered digestibility affects the nutritional impact of feed by reducing the availability of substrates to bacteria in the rumen. However, compositional data are used as an indicator of nutritive impact with non-GM varieties, hence direct animal feeding studies are not required. It is also important to note that there is no evidence from chemical analysis data or from studies with the PAT protein that T25 maize is unsafe for use as an animal feed.

Point 15. *There has been criticism of a digestion study carried out on the PAT protein*

It has been suggested that a digestion study involving the PAT protein did not accurately model the conditions likely to be found in animals eating the GM maize because the pH of stomach juices rises above the highest pH used in the study when food is ingested.

However, it must be recognised that acid digestion studies such as the one carried out here are only an indicator of how rapidly a protein might be degraded/ inactivated in the stomach and they are not designed, nor were they intended to be a detailed simulation of an animal's digestive system. ACAF was content that these studies supported the conclusion that PAT protein derived from oilseed rape and T25 maize is

inactivated *in vitro* by gastric juices. Measurements of relative PAT activity in simulated human gastric fluid also supports this conclusion³⁵.

Point 16. *It has been suggested that livestock show a preference for eating non-GM maize.*

ACRE has discussed the anecdotal nature of some of the concerns raised in the written representations and submissions for the CHARDON LL hearing. One claimed that some animals had shied away from eating GM crops. ACRE noted that it was difficult to answer these points in the absence of any tangible reason or mechanism for why this should be. ACRE was not aware of any peer reviewed scientific data but would welcome the submission of relevant papers or evidence at any time.

Further information

Detailed papers on the legal framework for GMO releases and the history and science of the Farm Scale Evaluations are available at:

<http://www.defra.gov.uk/environment/fse/index.htm>

The public register entry for T25 maize can be viewed at Defra during office hours Monday to Friday 0900-17.00hrs at the address below, or copies can be sent by first class post by phoning the public register enquiry line (see below).

The full T25 maize dossier (including 15 appendices) as submitted to the French authorities in 1995 and subsequent correspondence is also available for scrutiny on the same basis as the public register entry.

A report of ACRE's hearing, a verbatim transcript and the statements of witnesses invited to speak are also available on the ACRE website:
<http://www.defra.gov.uk/environment/acre/index.htm>

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³⁵ Wehrmann A., Van Vliet A., Opsomer C., Botterman J. and Schulz A. (1996). The similarities of *bar* and *pat* gene products make them equally applicable for plant engineers. *Nature Biotech.* **14**: 1274 – 1278.

Public register enquiry line: 020 7944 3409
Email: gm@defra.gsi.gov.uk

Alternatively you may submit further questions or comments by post or email to the above address.