ADVISORY COMMITTEE ON RELEASES TO THE ENVIRONMENT

Advice on a plant breeding technique involving oligo-directed mutagenesis: RTDS™

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Advice of the Advisory Committee on Releases to the Environment (ACRE) under S.124 of the Environmental Protection Act 1990 (Part VI) to the Secretary of State for Environment, Food and Rural Affairs, Scottish Ministers, Ministers of the Welsh Assembly Government and the Department of Environment (Northern Ireland).

Advice: ACRE considers that herbicide tolerant (HT) oilseed rape plants produced by Cibus LLC have been developed using a form of mutagenesis. It considers that this technique does not involve the use of recombinant nucleic acid molecules. Consequently, the HT oilseed rape plants could be excluded from the GMO Deliberate Release legislation in accordance with Annex 1B of Directive 2001/18/EC.

Introduction to oligo-directed mutagenesis

Oligonucleotide-directed mutagenesis (ODM) is a technique used to correct or to introduce specific mutations at defined sites of the genome. ODM is a generic term covering several approaches and applications. It is referenced in the literature under other names such as targeted nucleotide exchange, chimeraplasty, oligonucleotide-mediated gene editing, chimeric oligonucleotide-dependent mismatch repair, oligonucleotide-mediated gene repair, triplex-forming oligonucleotide-induced recombination, oligodeoxynucleotide-directed gene modification, therapeutic nucleic acid repair approach and targeted gene repair (see *e.g.* Andersen *et al*., 2002; Christensen *et al*., 2006; Cole-Strauss *et al*., 1999; de Semir and Aran, 2006; Igoucheva *et al*., 2006; Zhang *et al*., 1998).

All these techniques are based on the site-specific correction or directed mutation (base substitution, addition or deletion) of an episomal or chromosomal target gene after introduction of a chemically synthesized oligonucleotide with homology to that target gene (except for the nucleotide(s) to be changed). In all cases, the gene modification is induced directly and exclusively *via* the effect of the oligonucleotide itself, *i.e.* independent of any delivery vector system. The above-mentioned definitions do not cover cases where the oligonucleotide is chemically modified to incorporate a mutagen (the oligonucleotide is used as a vector to deliver the mutagenic agent in a DNA site-specific manner) (Kalish and Glazer, 2005), nor cases where the oligonucleotide is used together with zinc-finger nucleases (ZFNs) to generate double-strand breaks at specific genomic sites (Wright *et al*., 2005).

Although the usefulness of the technique has been first demonstrated in mammalian cells, preliminary studies at the end of the 1990s demonstrated that oligonucleotide-mediated mutagenesis is applicable to plants and can induce target gene mutations (Beetham et al., 1999; Gamper *et al*., 2000; Hohn and Puchta, 1999; Zhu *et al*., 1999). Successful *in vivo* gene modification has been demonstrated notably in maize, rice, tobacco and wheat, *e.g.* to create plants insensitive to the action of a specific herbicide (Dong *et al*., 2006; Iida and Terada, 2005; Kochevenko and Willmitzer, 2003; Okuzaki and Toriyama, 2004; Zhu *et al*., 2000). Altered genes have been shown to be stably maintained during mitosis (Beetham *et al*., 1999; Kochevenko and Willmitzer, 2003), and transmitted in a Mendelian fashion to subsequent generations (Zhu *et al*., 1999, 2000).

Herbicide tolerant oilseed rape produced by RTDS™

ACRE was requested to consider a specific ODM technique - the 'Rapid Trait Development System' (RTDS™) developed by Cibus LLC and used to generate oilseed rape tolerant to sulfonylurea herbicides.

Organisms produced by mutagenesis can be excluded from Directive 2001/18/EC (under which the release into the environment of genetically modified organisms are regulated) in accordance with Annex 1B of this Directive. This exclusion applies on the condition that the technique does not involve the use of recombinant nucleic acid molecules.

ACRE was asked to confirm whether RTDS™ should be considered a technique of mutagenesis along with more traditional techniques involving chemical mutagens and radiation (which are excluded from the Directive). In particular, it was asked to consider

whether the oligonucleotide (referred to as a GRON, Gene Repair Oligonucleotide in this context) that is used as a mutagen in the RTDS™ system is inserted into the host genome or persists in the cell rather than being present transiently.

The GRON that is introduced into plant cells is a chemically synthesised, single stranded oligonucleotide comprising DNA bases as well as non-naturally occurring chemical moieties. It was designed to hybridise to a specific fragment of one allele of the *ACETOLACTATE SYNTHASE* (*ALS*) gene in oilseed rape (*Brassica napus*). A one base pair change in the GRON compared with the target gene acts as a signal to the cell's repair mechanism to alter the corresponding nucleotide in the target gene. This one base pair change in the *ALS* gene confers tolerance to sulfonylurea herbicides in oilseed rape plants regenerated from cells in which this change was induced (*i.e.* there is a tissue culture step).

Once the alteration has been made, the oligonucleotide is degraded by the cell. This occurs rapidly in a matter of hours rather than days. In this respect, the GRON is equivalent to chemicals used in chemical mutagenesis *i.e*. cells are exposed transiently to these mutagens, which do not persist.

Cibus provided ACRE with information on the half-life of the GRON in plants and cell free extracts to support this conclusion. It also provided data to demonstrate the specificity of the GRON in introducing mutations at specific sites in particular genes. The weight of evidence is persuasive. However, it is extremely difficult to prove that no other changes of this magnitude (i.e. of one or a few base pairs) have occurred due to the use of this technique even by sequencing the whole genome (due to existing single nucleotide pair differences that occur naturally between individuals). However, traditional techniques of mutagenesis generate numerous indiscriminate changes to genomes. Ultimately, individuals with the desired mutation are selected and the vast majority of unlinked, undesirable mutations are lost through crosses with non-mutated plants. Mutation breeding of this type was first attempted in 1928 and has been a valuable breeding tool over the last 50 years . Varieties of crop plants generated through this traditional breeding technique include herbicide tolerant oilseed rape.

ACRE was also requested to consider whether it considered the GRON used in this technique a recombinant nucleic acid molecule. The committee concluded that it was not because the GRON is a synthetic molecule that does not combine two or more genetic sequences. On the contrary, it is identical to a fragment of one of the alleles of the *ALS* gene in oilseed rape with the exception of one base pair. ACRE noted that single nucleotide

polymorphisms (SNPs) such as this difference in the *ALS* gene are common in plants and occur naturally.

ACRE advises regulators that care is needed when communicating its conclusions on whether the products of certain mutagenic techniques should be regulated or not. In the introduction to this advice, ACRE has provided examples of different terms used to describe the technique and variations that may result in the insertion of genetic material that will result in new combinations of genetic material in the host plant. This advice from ACRE deals specifically with Cibus' RTDS™ technique.

ACRE also advises that continuing to adopt a process-based approach to regulation will lead to difficulties in the near future. The current legislation provides for the exclusion of organisms generated by mutagenesis. However, the criteria for exclusion mean that some products of mutagenesis using these new techniques could be regulated even though there is no credible scientific justification to differentiate between them and mutants that are not regulated. These organisms would not be distinguishable either between themselves or with the products of traditional mutagenesis, or indeed natural mutants.

This situation is likely to occur if regulators classify organisms for regulation based on their definition of a recombinant nucleic acid molecule. ACRE notes that when the current GMO deliberate release Directive was drafted, oligonucleotides (whether they are considered recombinant nucleic acid molecules or not) would not have been used in this way *i.e*. to confer a change without inserting genetic material into the host plant.

As discussed previously, another point for regulators to consider is that organisms developed through RTDS™ could not be distinguished at the molecular level from those developed through "traditional" mutation techniques (using chemical mutagens or ionizing radiations) or from wild-type organisms.

Detection and traceability are key aspects in the EU regulatory system on GMOs, in particular for GMOs used as Food or Feed (EC, 2003[1]). As a consequence, adequate molecular methods must be available that enable the detection and identification of each GMO individually (the so-called "transformation event"). Traditionally, identification is achieved by mapping a segment of DNA in the GMO corresponding to the junction areas, *i.e.* the regions where the transgenic DNA is inserted in the genome of the host organism. It

[1] Regulation EC 1829/2003 on genetically modified food and feed: http://ec.europa.eu/food/food/animalnutrition/labelling/Reg_1829_2003_en.pdf

is therefore important to realize that emerging techniques such as ODM that do not involve the introduction into the genome of foreign DNA sequences from other species would pose challenges for unambiguous detection and testing, and ultimately enforcement of the EU regulatory system.

Others have reached the same conclusion as ACRE. Breyer *et al*. (2009) used the same reasoning in concluding that organisms developed through ODM should not fall within the scope of the EU legislation. COGEM[2] is in agreement.

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[2] COGEM provides scientific advice to the Dutch government on the risks to human health and the environment of the production and use of GMOs and informs the government of ethical and societal issues linked to genetic modification.

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