Is regulatory oversight needed for field trials of plants produced using genome editing technology?

General Background

In developing improved crops, plant breeders have always depended on genetic variation within a population, to identify characteristics (genetic traits) or combinations thereof that offer an advantage to the crop. This variation is due to changes that can occur in the genetic sequence (DNA) of the crop plant over time – and is a normal part of evolution (mutations in the DNA). These changes can be responsible for many new characteristics such as plants with increased disease resistance or improved nutritional quality. Sometimes the desired variation is not available within a population, so breeders have used many different methods to introduce changes in the DNA, such as using chemical or physical (e.g. radiation) treatment of seed, to induce variation in the population. These methods have been widely used in conventional plant breeding for decades. These changes to the DNA sequence (referred to as 'mutations') often lead to a change that stops a particular gene from working. As well as being useful in plant breeding, mutations are incredibly useful as research tools. For instance, if a scientist can create a mutation in the gene that they are interested in, generating a non-functional gene (or a "gene knock-out") then they have a way of working out what that gene does, by looking at how plants with and without a functioning gene behave. Until recently, methods to introduce mutations were non-specific meaning they could target a large number of genes within the plant, including hopefully the DNA region, or gene, of interest. These mutant plants would then need several rounds of back-crossing to remove hundreds or thousands of unwanted mutations in many other genes, to end up with a plant quite similar to the wild-type or parental background, but retaining the desired mutation. There is a long history of familiarity with the process and the resulting plants can be tested in the field without regulatory over-sight. So far around 3,000 mutants have been released for commercial cultivation in the past decades and some of them have become widespread, such as the durum wheat variety Creso. Technology has now advanced and we have the ability to create plants with mutations in genes-of-interest in a more precise and controlled manner using genome editing. One specific genome editing technology, called CRISPR/Cas9, is currently gaining rapid interest within the research community because of its precision and user-friendliness.

So it is now possible to introduce mutations into plants in a number of different ways to get to the same end point, for example a plant that lacks a particular functioning gene.

In the exercise below we describe the production of five *Arabidopsis* mutant lines (A to E, see also supplementary details that follow), all lacking the function of the same photosynthetic protein, PsbS, that is a component of the photosynthetic machinery. For 4 of the 5 mutations the regulatory situation for growing these plants in the field is clear. By considering whether or not they contain foreign DNA we can see whether they fall within the scope of the EU directive 2001/18/EC. However, for 1 of the 5 mutants the regulatory path remains unclear. We seek to ask the same question to different regulatory authorities; 'where do you think plant E should sit?' Do you feel this plant would need or would not need regulatory oversight for field testing?

Mutant	Mutation achieved by	Is regulatory oversight under the 2001/18 EC directive needed
A	Radiation Breeding	No (contains no foreign DNA)
B	Chemically induced	No (contains no foreign DNA)

C	T-DNA knock-out	Yes (T-DNA present)
D	CRISPR/Cas9 first generation plant	Yes (T-DNA present)
E	Plant derived from D above, with the CRISPR/Cas9 T-DNA segregated out. Sequence analysis shows the plant to be identical to the wild-type with the exception of the single gene mutation. No foreign DNA is present.	? (contains no foreign DNA)

Several "genome editing techniques" have been developed over the last decade; the most powerful is named CRISPR/Cas9. The regulatory status of these – sometimes classed as New Breeding Technologies (NBTs) – in the EU is unclear, and the scientific community has been waiting several years for clarification from the EU. A key issue is that the changes introduced by NBTs may be indistinguishable from those introduced by other techniques where the resulting plants are not regarded as GMOs. This raises serious issues of legal certainty: as the resulting plants contain no foreign DNA, it is impossible to tell forensically how they were derived and therefore whether the person growing them is abiding by the regulations should the plants be included within the scope of the EU directive 2001/18/EC.

A critical issue in the EU directive 2001/18/EC is the definition of a GMO; a GMO contains "genetic combinations that would not occur naturally by mating and/or natural recombination". Organisms that do not fall under this definition are exempted from the legislation. In the case of NBTs like CRISPR, the genetic combinations are identical to those that could occur naturally, and in our opinion these have to be exempt.

Supplementary details (see also attached pdf)

To illustrate this issue, we look at how the same gene in *Arabidopsis thaliana* has been mutated using five different methods, each resulting in a loss of function (mutation) of the same gene. The gene is called PsbS – plants which lack this protein have reduced fitness and poor seed production.

A. Radiation breeding has been used by geneticists since the 1920s. It uses either X-rays, gamma rays or fast neutrons. Mutant A (npq4-1, Li et al. 2000) lacking PsbS was identified from a collection of fast neutron-mutagenized seeds, obtained from a commercial supplier. The use of radiation leads to a deletion of DNA, in this case the whole PsbS gene and some surrounding genetic material. Other genes may have also been altered and this effect is unknown despite this mutant being studied extensively. Regulatory oversight is not needed for plants developed from this method.

B. Chemical mutagens have been used since the 1940s. Ethyl methanesulfonate (EMS)-induced mutagenesis is the most popular, and Mutant B (npq4-4, Li et al. 2000) lacking PsbS was identified from a collection of EMS mutagenized seeds, generated in an academic lab. EMS mutagenesis leads to thousands of point mutations; single bases (T, G, C or As) in the DNA being changed to another. Changing these single base pairs leads to a disruption in the gene and loss of function. Regulatory oversight is not needed for plants developed from this method.

C. Mutagenesis induced by insertion of the so-called T-DNA (Transfer-DNA) of *Agrobacterium tumefaciens.* The location where the T-DNA lands is non-specific, but as hundreds of thousands of T-DNA inserts have been generated across the genome of Arabidopsis, T-DNA knock-outs of most genes have been generated. This leads to interruption of the coding region of the gene, hence no protein is produced. Many independent lines with insertion in the PsbS gene can be identified in public databases and are freely available at stock centres for research purposes. This method is subject to regulatory oversight as DNA from the *Agrobacterium* (including anything within the T-DNA and potentially additional vector backbone sequence) is, or could be, present in the resulting plant.

While the mutations described above have been generated in the lab, it is of note that natural mutations have also occurred spontaneously in this way in nature over thousands of years. This has been reported recently when the sweet potato genome was sequenced and T-DNA from *Agrobacterium* was found to be present in the plants genome.

D. CRISPR/Cas9-induced genome editing, whereby an introduced nuclease (Cas9) can be targeted to 'cut' specified locations in the genome by also introducing a guide RNA sequence to target the required gene. When two target guide RNAs are used it can lead to two simultaneous cuts within the gene to generate deletions (like in mutant A) or when a single target guide RNA is used, a single cut in the gene will result in point mutations (like in mutant B). In our case, a T-DNA coding for Cas9 and two guide RNAs each targeted to two separate sites in the PsbS gene, was introduced by *Agrobacterium-mediated* transformation. Once in the plant, these induce a double stranded break (cuts) of DNA at the target sites, leading to a deletion of the genetic material between the sites. Deletions (or point mutations) induced by CRISPR/Cas9 are not different from those induced by radiation or EMS. However, in the primary transgenics the T-DNA remains; and the plant is considered a GMO. It is therefore subject to regulatory oversight.

E. Some offspring from the plants produced from method D above will inherit the mutation but a percentage will not inherit the T-DNA with Cas9 and the guide RNAs. Hence, these plants cannot be distinguished from those induced by radiation or EMS (except that EMS mutations also lead to many mutations elsewhere in the genome). Sequence or PCR analysis of E can be undertaken to confirm that no T-DNA sequence remains.

According to scientific logic, mutants A-E are equivalent since they lack the same protein. By regulatory logic, these are very different due to their route of production. The question we set out to ask is should mutant E be exempted from the 2001/18 directive in the same way that Mutant A and B are?

References

Li, X-P, Björkman, O, Shih C, Grossman, AR, Rosenquist, M, **Jansson, S,** Niyogi, K.K. (2000) A pigment binding protein essential for regulation of photosynthetic light harvesting. **Nature** 40: 391-395.

Külheim C, Ågren J, **Jansson S** (2002) Rapid regulation of light harvesting is crucial for plant fitness in the field. **Science** 297:91-93.