Advisory Committee on Releases to the Environment (ACRE)

Advice on an application for deliberate release of a GMO for research and development purposes

Applicant: Rothamsted Research

Application: field assessment of ultra-low asparagine, low acrylamide, gene edited wheat.

Ref: 21/R08/01

Date: July 2021

Advice of the Advisory Committee on Releases to the Environment to the Secretary of State under section 124 of the Environmental Protection Act 1990

ACRE is satisfied that all appropriate measures have been taken to avoid adverse effects to human health and the environment from the proposed release. ACRE sees no reason for the release not to proceed according to the following advice.

To minimise the likelihood that Genetically Modified (GM) wheat from this trial will enter the human food or animal feed chains, the applicant should:

1. Ensure that the 20m surrounding the trial site is planted with a non-cereal crop and that cereal volunteers are controlled (prior to flowering) in this area during the trial.

2. Plant a wheat pollen barrier of 3m width, to flower at the same time as the GM wheat as an additional precautionary measure.

3. Control *Elytrigia repens* (Couch Grass) using a glyphosate herbicide and hand-weeding if necessary, within the trial site and the surrounding 20m, before flowering and for the duration of the trial.

4. Ensure that any GM or non-GM wheat plant material remaining in the area of release at the end of the trial is disposed of appropriately.

5. Ensure that following harvest, the area of release is lightly tilled twice (once after harvest and again in the following spring) to a depth of 5cm to stimulate germination of any wheat plant volunteers. The release areas should be left fallow and monitored for wheat plant volunteers for 2 years following harvest.

6. Record the number of wheat plant volunteers that germinate before destroying them with an application of glyphosate herbicide or hand pulling them prior to flowering.

7. Ensure that suitable measures (such as those described in Rothamsted Research's application) are put in place to keep large birds out of the trial area and that the efficacy of these measures are kept under review.

8. Ensure that machinery used on the site is cleaned thoroughly onsite, including between using it with GM and non-GM material, and that clothing and equipment such as vehicles used by personnel on the site are also cleaned thoroughly before leaving the site.

Comment

ACRE considered the risks to human health and the environment posed by the proposed release of wheat that has been gene edited to accumulate ultra-low levels of asparagine in its seeds. The aim of this research is that lower asparagine in the grain results in lower acrylamide accumulation when heated during food production processes.

Under glasshouse conditions, these gene-edited plants had significantly lower germination levels than the wild type.

The primary purpose of this trial is to examine further the agronomic characteristics of this gene-edited wheat that are altered under field conditions.

A second aim is to complete segregating out of the 3 transgenes that had been introduced to carry out gene editing, but which are now no longer required.

Key characteristics of this field trial with respect to its environmental risk assessment are:

i) It will be on a small scale. This application is to release approximately 300 seeds per m^2 over an area of 1470m^2 comprised of 50 plots each of 6m by 1.8m, of these only 25 will contain gene-edited plants. The applicant has proposed that the release will take place at different locations within their GM trial site over 5 years.
ii) The trial will be planted first in the autumn of 2021 and then sequentially until the final harvest in Summer 2026. Autumn-sown plants will be harvested in August or September of the following year.
iii) The GM wheat and non-GM wheat grown in this trial will not be put into the human food chain or fed to livestock.

The applicant intends to trial 5 gene-edited lines, all contain point mutations (or deletions) within the first exon of at least 1 homeologue of a gene encoding asparagine synthetase: *TaASN2*, which is expressed in a seed specific manner.

The production of these gene edited plant lines required genetic modification to introduce 3 transgenes, these genes were carried on 3 separate plasmid vectors and were co-transformed into cultivar (cv.) Cadenza wheat embryos.

Further, it was noted by ACRE that the applicant reports the segregation of these transgenes is not yet complete, such that 2 out of the 5 lines will still contain the clustered, regularly interspaced, short palindromic repeats (CRISPR) associated protein (Cas) 9 gene, along with the guide RNA (gRNA) and the selective marker. ACRE did not conclude that this created concerns with regards to risk assessing any environmental impact.

Molecular characterisation

ACRE noted that the plants for this trial were made using current gene editing tools in a UK milling winter wheat variety cv. Cadenza. It was edited using the (CRISPR) system coupled with the Cas9 nuclease to target 1 of the genes encoding asparagine synthetase: *TaASN2*.

The latter is expressed in a seed specific manner, where levels of free asparagine in the grain potentially lead to formation of acrylamide during high temperature food production processes.

The production of the gene edited plants required genetic modification to introduce 3 transgenes:

- a gene to express a total of 4 gRNAs targeting the TaASN2 gene homologues
- a gene to express the Cas9 nuclease itself, and a bar marker gene encoding tolerance to phosphinothricin based herbicides

These genes were carried on 3 separate plasmid vectors and were co-transformed into the scutella of Cadenza wheat embryos by microprojectile bombardment.

The application contains sequencing data for a total of 5 plant lines of the transgenic second (T2) generation, all of which will be grown in the trial. All have either biallelic or homozygous point mutations (or deletions) within the first exon of at least 1 homeologue of *TaASN2*.

Furthermore, some of these mutations or deletions result in extensively truncated proteins, whilst 2 of the lines lack the key glutamine binding domain.

Public representations questioned whether the term gene knock-out, as used by the applicant, was the correct description for an extensively truncated gene expression product.

Such discussion is beyond the remit of this advice paper, suffice to say a shortened expression product, or 1 that is otherwise modified such that in either case it lacks a key active site in the resultant polypeptide is considered to be biologically inactive[1].

ACRE noted that there was a continued presence of the transgenes used in the gene editing process, in at least some of the 5 lines. Polymerase chain reaction (PCR) assays using genomic DNA from T3 generation plant material, revealed that in 2 out of the 5 lines, the Cas9 gene, along with the gRNA, was still present in the plants producing the seed intended for sowing.

Additionally, at least 1 or other, or both, of the remaining 2 introduced genes (bar and the gRNA) were also still present in some plants across all 5 lines. There are several recent

[1] Voytas, D. F. and Gao, C. (2014) Precision genome engineering and agriculture: opportunities and regulatory challenges. *PLoS Biol.* **12**, e1001877.

reviews on the elimination of editing mechanisms in gene-edited non-transgenic plants: Sheng et al., 2020, Michno et al. 2020 and He et al. 2019[2, 3, 4].

Public representations alluded to the presence of transgenes and questioned why the applicant had not looked at the stability of these inserts. A previous release of gene-edited plants had similarly not looked at the stability of inserts, because there was the similar intention to segregate out the transgenes.

ACRE considered the continued presence of transgenes in gene-edited plants in their advice on this previous deliberate release field trial (See 10R5201).

In that instance the Cas9, gRNA and selective resistance genes were all introduced into Chinese Kale in a single plasmid vector via *Agrobacterium tumefaciens* mediated transformation.

However, the application contained no data on the continued persistence of these genetic elements in the resultant gene-edited plants, nor had they considered the likelihood of any off-target gene editing events. ACRE advised that the applicant should therefore screen all their plant lines for the presence of these transgenes prior to planting.

By contrast the present proposed field trial has provided screening data on the presence of transgenes involved in the gene-editing process, and in recognition of the segregation process specifically includes the elimination of these as 1 of the trial's initial aims.

To this end the applicant intends that a selection of plants will be analysed before flowering and the heads of plants that are shown to be transgene-free will be bagged to ensure self-pollination, so that seeds can be collected for subsequent sowing.

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[2] Sheng X, Sun Z, Wang X, Tan Y, Yu D, Yuan G, Yuan D and Duan M (2020) Improvement of the Rice "Easy-to-Shatter" Trait via CRISPR/Cas9-Mediated Mutagenesis of the qSH1 Gene. Front. Plant Sci. **11**:619. doi: 10.3389/fpls.2020.00619

[3] Michno, JM., Virdi, K., Stec, A.O. et al. Integration, abundance, and transmission of mutations and transgenes in a series of CRISPR/Cas9 soybean lines. BMC Biotechnol **20,** 10 (2020). https://doi.org/10.1186/s12896-020-00604-3

[4]He Yubing, Zhu Min, Wang Lihao, Wu Junhua, Wang Qiaoyan, Wang Rongchen, Zhao Yunde, Improvements of TKC Technology Accelerate Isolation of Transgene-Free CRISPR/Cas9-Edited Rice Plants, Rice Science, Volume 26, Issue 2, (2019), Pages 109-117, https://doi.org/10.1016/j.rsci.2018.11.001. (TKC= Transgene killer CRISPR)

The environmental risk assessment

The major trait in the plants is reduced accumulation of free asparagine in the grain. This appears to have a negative effect on germination, and there is some evidence of increased seed size in some of the edited lines.

Otherwise, the phenotype of the edited lines, including morphology, pollination, and seed-set, do not appear to differ from control wheat cv. Cadenza plants.

Therefore, it is expected that dissemination of pollen and seeds will be the same as for non-transgenic wheat plants, and the survivability of the plants in unmanaged systems will be reduced due to the effect on germination.

The applicant does not expect the gene-edited lines to differ from conventional wheat in terms of their capacity to self or cross pollinate via sexual reproduction. Therefore, the applicant anticipates a low rate (approximately 1%) of cross pollination with closely adjacent wheat plants within the trial.

Wheat is naturally self-pollinating but under experimental conditions can be crossed with various wild grasses. The application discusses sexual compatibility with wild relatives present at the trial site.

Elytrigia repens (common couch) is the only 1 of these common on the Rothamsted estate and it is proposed to control it and other grasses and weeds in and around the larger GM trial site either by applying non-phosphinothricin herbicides or hand pulling.

No cereals or grass species will be allowed to grow within 20 m of the trial area itself. It should be noted that the applicant reports that no spontaneous hybrids between wheat x *Elytrigia* have been found.

ACRE concluded that there was nothing about the intended phenotype of the wheat in the proposed trial of ultra-low asparagine and low acrylamide that may add additional risk.

Moreover, depending upon the weather conditions, the crop would be at risk from disease without chemical control and the applicant appeared to have accepted this risk.

The applicant notes that it is conceivable that editing could continue in plants in which the Cas9 gene is still present and assumes, based on published data that where present, (Miroshnichenko, 2019)[5], this and the other transgenes are integrated into the plant's genomic DNA.

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[5] Miroshnichenko, D.N., Shulga, O.A., Timerbaev, V.R. *et al.* Achievements, Challenges, and Prospects in the Production of Nontransgenic, Genome-Edited Plants. *Appl Biochem Microbiol* **55,** 825–845 (2019). https://doi.org/10.1134/S0003683819090047

ACRE were mindful of the consideration given to off-target effects arising from the continued presence of similar transgenes described in the Chinese Kale trial as alluded to above. In that trial, the applicant considered there was minimal risk of these plants having a greater negative environmental impact as a result of Cas9 cutting a DNA sequence that is similar to that of the target.

ACRE agreed that it was highly unlikely that the crop's potential for invasiveness and persistence would change as a result of additional mutations. In addition, the plots in which the gene edited plants will be grown and the area surrounding these plots will be monitored during and after the trial.

Furthermore, as described below measures to minimise seed survival on the site and cross-pollination with sexually compatible species will also be put in place as a precaution.

ACRE's opinion was that although loss by segregation of the transgenes was not yet complete in some of the seed to be sown in this trial, the committee did not see any problem with this in particular with regard to environmental impact.

ACRE's view was that the continued presence of the transgenes had been adequately assessed in the environmental risk assessment particularly when the afore-mentioned consideration of how able wheat is either to cross pollinate or out cross with wild relatives.

The committee noted that the proposer described further in their application that a selection of plants will be analysed before flowering and the heads of plants that are shown to be transgene-free will be bagged to ensure self-pollination, so that seeds can be collected for subsequent sowing.

ACRE noted previously that traditional mutagenesis techniques used in plant breeding generate many hundreds of off-target effects. The majority of these are lost when the mutant plants with desired characteristics are 'backcrossed' to lines that have not been mutated.

There is within the application an assessment of the likelihood of horizontal transfer of these transgenes, along with consideration given to recombination with soil bacteria, these are considered now.

The bar gene encodes phosphinothricin-N-acetyl transferase (PAT) protein that confers tolerance to glufosinate ammonium herbicides. This herbicide-tolerant trait was used as a selectable marker in identifying GM plants during the development stage of this project.

The herbicide will not be used in the trial. However, control by herbicides based on other active ingredients will not be affected.

ACRE considered in previous advice the possible selective advantage conferred on those plants that retained the glufosinate tolerance trait. In addition, a number of public representations also raised the matter of the potential selective advantage on these plants.

The applicant clearly sets out that this herbicide was used in the initial screening process to select for the presence of the co-transformed plasmids in wheat cells and plants during the development of these gene-edited plants.

Glufosinate ammonium herbicides will not be used at the trial site. ACRE also noted that genes encoding the PAT protein (which confers tolerance to glufosinate ammonium herbicides) are already widely present in soil bacteria and also that glufosinate herbicides are no longer permitted for field use in the UK.

Approximately 20 GMOs have been authorised for feed and food use in the EU, which produce the PAT protein (which confers tolerance to glufosinate ammonium herbicides). In each case, the conclusion of the European Food Safety Authority's (EFSA's) safety assessment was that these GMOs are unlikely to pose a greater risk to human health or to the environment than their non-GM counterparts.

As part of this assessment, EFSA has considered the toxicity and allergenicity of the PAT protein. This is reflected in Rothamsted research 's application such as, that the enzymatic function of PAT is specific to its substrate phosphinothricin, which does not occur naturally in humans.

PAT is degraded and inactivated in simulated gastric fluid and is therefore unlikely to retain any enzymatic activity in vivo. No sequence homology between the PAT protein and known toxins had been found. The native PAT protein (51% purity) has been tested for acute toxicity in mice and no toxicity has been reported at a dose of 5g per kg body weight.

The plasmids that were used for the transformation process possess a bacterial origin of replication and antibiotic resistance marker genes, and the applicant states these plasmids are still present to a greater or lesser extent in the plants being used to produce seed for the first year of the field trial.

The applicant further states that it is reasonable to assume that these elements are integrated into the plant genomic DNA. These elements provide a theoretical mechanism for homologous recombination with soil bacteria and positive selection if relevant antibiotics are present.

However, the proposer estimated the probability that horizontal gene transfer could occur to be extremely low, and the risk represented by such transfer must be seen in the context of these genetic elements already being present in soil bacteria.[6]

[6] Walsh F, Duffy B (2013) The Culturable Soil Antibiotic Resistome: A Community of Multi-Drug Resistant Bacteria. PLoS ONE **8**: e65567.

ACRE gave the following advice on plant to bacterial gene transfer in a previous field trial application:

Even though the scientific consensus is that selection pressure on bacteria containing antibiotic resistance genes is the driver of antibiotic resistance gene frequency in the environment.

ACRE discussed the potential for bacteria in the environment to be transformed with antibiotic resistance genes from the gene edited wheat plants. Studies of horizontal gene transfer from plants to bacteria suggest that this phenomenon is extremely rare (refer to a review by Keese, 2008[7]).

ACRE noted that even if a recombination event were to occur between DNA from a plant and a bacterial genome, in order for the gene to be expressed, it would need to be combined as a fully functional transcription unit in the bacterium, which is unlikely. If it were to occur, it would most likely result from a homologous recombination event at a site in the bacterial genome where a version of antibiotic resistance gene already exists.

This transfer potential also applies to the Cas9 gene, if homologous recombination were to occur it would be with a similar Cas system already present in the soil bacterial genome.

Therefore, without the guide RNA needed to target its nuclease function, there would be little selection pressure to retain it in the recipient genome. No toxic, allergenic or harmful effects on human health are envisaged by the proposer.

ACRE noted that, on the contrary, the aim of lowering the free asparagine concentration in the grain is to improve food safety by reducing the potential for acrylamide to form during baking, toasting, and processing. Acrylamide is a Class 2a carcinogen, causes birth defects and has neurotoxic and anti-fertility effects at high doses.[8]

A number of the public representations recommended that toxicity and allergenicity studies should be carried out. This is not generally necessary for small-scale trials where material will not enter the food or feed chains unless there is a plausible hypothesis whereby such limited exposure to the plant material could cause harm to humans and other animals.

The consideration of any altered allergenicity if this crop were to be used in food production is therefore beyond the remit of ACRE.

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[7] Keese P. (2008). Risks from GMOs due to horizontal gene transfer. Env Biosafety Research. **7**(3): 123 – 149

[8] CONTAM Panel (2015) Scientific opinion on acrylamide in food. *EFSA Journal* 13: 4104.

Managing the trial site

ACRE has considered the potential risks of this trial to human health and the environment in the context of it being a small-scale trial from which no material will enter the food or feed chains, the committee considered, in detail, management plans to minimise the persistence of GM material at the trial site and the dispersal of GM material from the site.

ACRE recognised that, although the trial is larger than a previous wheat trial in terms of area, it was still small and therefore a 3m pollen barrier and a 20m isolation distance was adequate to minimise the probability of out-crossing to an acceptable degree.

Gene flow

Wheat is a self-pollinating crop with very low rates of cross-pollination with other wheat plants. This is because fertilisation often occurs before the florets open, which makes out-crossing unlikely, in addition, wheat pollen is relatively heavy and tends to travel shorter distances than pollen from other grass species that are wind-pollinated.

Studies have detected cross-pollination rates of 1 to 2% between wheat plants in close proximity, but this rapidly decreases with the distance between plants. There are several relevant studies involving GM wheat field trials, most recently those of Foetzki *et al*. (2012)[9] and Miroshnichenko *et al.* (2016)[10].

The area for the proposed trial will comprise 50 plots of 6m by 1.8m and will be sown at a density of 300 seeds m^2. There will be 1m separation between plots and a wheat pollen barrier of 3m width entirely surrounding the trial plots, with a further 20m surrounding that, in which no cereals or grass species will be left to grow.

ACRE sought clarification from the applicant as it was not clear exactly what the actual experimental arrangement was within plots. The response was to confirm that each plot will contain a single line selected for that plot using a randomised design protocol.

ACRE noted that the separation distance required to prevent hybridisation between different wheat varieties when certified seed is produced for marketing purposes is 2

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[9] Foetzki A., Diaz Quijano C., Moullet O., Fammartino A., Kneubuehler Y. and Mascher F. (2012). Surveying of pollen-mediated crop-to-crop gene flow from a wheat field trial as a biosafety measure. GM Crops and Food: Biotechnology in Agriculture and the Food Chain **3**(2), 115–122.

[10] Miroshnichenko D., Pushin A and Dolgov S (2016). Assessment of the pollen-mediated transgene flow from the plants of herbicide resistant wheat to conventional wheat (*Triticum aestivum* L.). Euphytica **209**:71–84.

metres. The application proposes to sow a 3m wide wheat pollen barrier (comprising the same variety as the GM wheat) around the trial.

ACRE recommended a 2m wide pollen barrier in its advice on 2 previous GM wheat trials at the same site as this is an additional precautionary measure to the 20m separation distance.

But, recognising the larger size of this trial, ACRE sees no reason not to extend this to 3 metres to ensure an acceptable probability of no unacceptable gene flow. In order to maintain the separation distance.

ACRE advises that the 20m surrounding the trial site is planted with a non-cereal crop and that cereal volunteers are controlled (prior to flowering) in this area during the trial and for 2 years afterwards.

ACRE members considered that in terms of the pollen barrier, the key was timing to make sure both the experimental crops and the pollen barrier crop were at the same stage of development. That can be difficult if 1 is looking at experimental seed that does not have all the characteristics and stability of a commercial variety.

The committee concluded that, in their view, if synchronisation proves difficult, then the 20m separation distance would be an acceptable risk mitigation.

The applicant plans to move the specific site around within the bounds of the Rothamsted GM field trial site to allow post trial monitoring and to avoid the effects of take-all disease on the plants.

Therefore, because the whole site is enclosed by a fence to prevent unauthorised access, the location of the specific growing site will require careful consideration to ensure that the 20m isolation distance remains within the fenced-off site as a whole.

Wheat plant volunteers

The trial will receive standard farm practice as regard to herbicides (except that phosphinothricin based herbicides will not be used), fungicides, nitrogen, sulphur, and other fertilisers.

The site will be monitored regularly (at least weekly) both during and for 2 years after the trial. For the post-trial monitoring period, the trial area will remain in stubble to enable monitoring of volunteers.

The applicant does mention shallow cultivation being carried out to encourage volunteers but does not specify when this will be done.

ACRE advice on previous trials has been lightly till twice (once after harvest and again in the following spring) to a depth of 5cm to stimulate germination of any wheat plant volunteers. The persistence of such volunteers from winter wheat in cultivated soil has been studied for a long time and is well-characterised. [11],[12]

In common with previous GM wheat field trial applications, public representations have raised the issue of volunteers found by The United States of America Department of Agriculture, Animal and Plant Health Inspection Service (USDA APHIS) in 3 states some years after trials had been carried out.

There had been more than 100 large-scale trials conducted across 16 states, and although USDA APHIS's investigations were inconclusive, it was as previously discussed by ACRE, most probably due to the persistence of volunteers that GM seed had mixed with other seed and in this way become spread to other fields.

This trial is on a very small scale and has a number of measures, including post trial monitoring, to ensure that any volunteers are detected and removed.

Seed movement

ACRE were content with the applicant's outline of how the release will be monitored regularly during all stages of development and harvested at maturity. Some seeds from the GM and control plots will be conditioned, threshed, and stored in appropriate GM seed stores.

A sample of plants may be hand-harvested, conditioned and threshed to supply seeds for research purposes. All such small samples removed from the trial site will be stored in containment prior to use and will eventually be autoclaved before disposal. The remainder of the site will be harvested by the plot combine.

Laboratory analysis will assess the effect of low grain asparagine concentration on grain yield, grain size (thousand grain weight), grain protein content and quality, the concentrations of other free amino acids, total seed nitrogen and sulphur, Hagberg Falling Number, starch, and sugar content.

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[11] Chepil W.S. (1946) Germination of Weed Seeds I. Longevity, Periodicity of Germination, and Vitality of Seeds in Cultivated Soil. Scientific Agriculture **26**: 307-346.

[12] Anderson, R. L., and G. Soper. 2003. Review of volunteer wheat (Triticum aestivum) seedling emergence and seed longevity in soil. Weed Technol **17**:620–626.

The formation of acrylamide in heated flour produced from the grain harvested from the trial will also be measured.

Grain that is not required for analysis or to provide seed for future trials and all other material, including that from the pollen barrier rows, will be disposed of by incineration, autoclaving, or deep burial at a local authority approved landfill site using an approved contractor, while any material remaining after analysis will be autoclaved before disposal.

Transportation of waste materials will be in secure containers. All straw will be chopped and left on site. The combine will be cleaned prior to leaving the site so that all traces of plant material from the trial will remain in the trial area. All transport of material will be logged.

Other items arising from public representations

Some 95 public representations were received, where these covered areas within the remit of ACRE they were addressed within its assessment, as summarised above. Many of the representations concerned areas beyond the remit of ACRE, and/or these topics are not relevant to the environmental risk assessment when considering a small-scale field trial.

Some of these comments would be of relevance if the application had been for commercial-scale cultivation and/or food and feed use.

Others were more political in nature and are outside of ACRE’s remit, for example, they questioned whether GM techniques should be used in the development of plant varieties and more specifically whether it is necessary to develop wheat with reduced asparagine using this technology.

They also questioned whether this was the best way to reduce acrylamide in wheat flour, a point covered by the applicant who entertains that this gene edited wheat is only 1 possible contributory element to this aim.