Advisory Committee on Releases to the Environment

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

Applicant: University of Oxford

Application: Improving yields and stress tolerance in wheat by using CHLORAD as a technology

Ref: 24/R57/01

Date: December 2024

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 GM wheat from this trial will enter the human food or animal feed chains, the applicant should:

1. Ensure that there is 20m surrounding the trial site, in which no cereals or grass species will be left to grow, other than those being trialled under separate GM releases.

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

3. Control *Elymus repens* (Couch Grass) and *Elymus caninus* (Bearded couch) using hand-weeding, other mechanical methods or application of glyphosate herbicide, within the trial site and the surrounding 20 m, 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 5 cm 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 hand pulling, mechanical methods (e.g. harrowing) or application of glyphosate herbicide prior to flowering.

7. Ensure that suitable measures (such as those described in the University of Oxford'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.

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 with respect to chloroplast-associated protein degradation (CHLORAD)[1]. The primary purpose of this trial is to examine the agronomic characteristics of this gene-edited wheat under field conditions, especially improved yields and stress tolerance of wheat.

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

i) This application is for a one-year trial of GM wheat cv. Fielder, with the planting of the first crop in Spring 2025. Harvesting is planned for August/September 2025. The trial will be conducted at four sites in England.
ii) The maximum area for the proposed trial at each site will be 400m^2, including both GM and controls, spacing between plots and the pollen barrier. A maximum of 30 plots being *SP1* gene-edited GM lines. The maximum number of GM plants released per trial site will be 7500.
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.

There are nine gene-edited plant lines planned to be trialled, all with null levels of *SP1*, and which are homozygous for this in at least one sub-genome. Eight of these lines still retain the transgenic construct that was transformed into the plants to perform the gene editing. This transgenic construct included the plant selection resistance gene for hygromycin (*hptII*), the CRISPR/Cas9 and the two single guide (sg) RNA specific for the *SP1* gene.

Molecular Characterisation

ACRE noted that the plants for this trial were made using current gene editing tools in a spring wheat variety cv. Fielder. They were edited using the clustered, regularly interspaced, short palindromic repeat (CRISPR) system coupled with the Cas9 nuclease[2], to target a master regulator of chloroplast protein import, *SP1*.

The *SP1* gene is a key component of the CHLORAD system[1], which is itself a master regulator of chloroplast protein import. Mutations in *SP1* can lead to traits of interest in new, more stress tolerant crop varieties, and in particular delayed leaf senescence[3,4]. This results in a stay-green phenotype because it is associated with prolonged photosynthetic activity, and potentially improves yield, tolerance to stresses and disease resistance.

The production of these plants involved genetic modification using *Agrobacterium tumefaciens* mediated transformation to incorporate a cassette construct. This contained the plant selection resistance gene for hygromycin (*hptII*), the CRISPR/Cas9 and the two sgRNA specific for the *SP1* gene. All were under the control of separate plant specific promoters.

There are nine gene-edited plant lines planned to be trialled, all with null levels of *SP1*, and which are homozygous for this in at least one sub-genome. However, eight of these lines still retain the transgenic construct that was transformed into the plants to perform the gene editing. The construct was detected by a PCR assay that looked for the *hptII* gene and then confirmed by sequencing of the amplicon. Other elements within the construct were not assayed. There are several recent reviews on the elimination of editing mechanisms in gene-edited non-transgenic plants[5,6,7].

The seed for this trial is from either the T4 or T5 generation, depending on the plant line. ACRE noted that the *hptII* marker gene was detected across generations of the transformed plant lines, strongly suggesting that it is inserted in the nuclear genome in a stable manner. The nuclear location was not determined for the transgene, nor was its copy number. This marker will not be utilised in the proposed field trials. Genotyping of progeny lines indicated that no further edits were made despite the continued presence of the gene editing cassette.

The Environmental Risk Assessment

ACRE's considered view was that this release presented negligible risk to the environment and human health and further, that the continued presence of the transgenes had been adequately assessed in the environmental risk assessment. The

committee noted that there was also suitable consideration of both cross pollination and out crossing of the wheat undergoing trial with that of wild relatives; and ACRE were content that the applicant had put in place suitable measure to reduce this further.

Stay-green phenotype

The major trait in the plants is the stay-green phenotype, which results in prolonged photosynthetic activity, and potentially improves yield, tolerance to stresses and disease resistance. In all other ways, gene-edited lines were indistinguishable from un-edited control lines under controlled environmental growing conditions. The applicant also stated that within each edited plant line there was no phenotypic variation among individuals, and that the selected lines grew consistently. This was based on observations on morphology, flowering time, pollination and number of tillers of plants under glasshouse conditions.

ACRE concluded that the stay-green trait in wheat would not increase the environmental hazard compared to non-GM plants. Any increase in grain yield could theoretically increase persistence, because the increased grain number and viability per square metre could increase the chance of volunteers. ACRE stated that a commercially relevant yield increase of a few percent would not significantly alter that hazard.

ACRE noted the potentially improved stress tolerance/disease resistance seems possible but had not been tested. They concluded that even if realised, its indirect effect on invasiveness and persistence would likely be modest at best.

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 sites. *Elymus repens* (common couch) is the only one of these common on the four trial sites, with *Elymus caninus* (Bearded couch) also present at two of the trial sites. ACRE advise that Common couch, Bearded Couch, other grasses and weeds are controlled in and around the larger GM trial sites by hand pulling, mechanical methods (harrowing) or applying glyphosate herbicides. No cereals or grass species, other than those being trialled under separate GM releases, will be allowed to grow within 20m of the trial area itself. It should be noted that the applicant reports that no spontaneous hybrids between wheat *x Elymus* have been found.

CRISPR/Cas9

The applicant has assessed for potential off target edits using the WheatCRISPR tool[8] and found that none were expected. By genotyping progeny lines, the applicant has observed the gene edits were stable and there were no further edits in *SP1* homoeologues in subsequent generation(s). This suggests the Cas9 system was non-functional, even on its intended target genes. Nevertheless, ACRE considered the effects of off-target edits arising from the continued presence of the Cas9 transgene. As in previous GM deliberate release trials (See 10/R52/01 & 21/R08/01), ACRE concluded that it was highly unlikely that the crop's potential for invasiveness, persistence or environmental risk would change as a result of additional off-target mutations. ACRE concluded that the presence of the Cas9 is an extremely low risk, given that the guide RNAs have high predicted specificity.

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.

In addition, the plots in which the GM 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.

Hygromycin resistance

The *hptII* gene encodes for hygromycin resistance, an antibiotic tolerant trait which was used as a selectable marker in identifying GM plants during the development stage of this project. ACRE noted the selectable marker, *hptII*, includes an intron which restricts its expression to eukaryotic hosts. Furthermore, the *hptII* gene is among The European Food Safety Authority's (EFSA) Group 1 antibiotic resistance marker genes (ARMGs) of least concern in GM plants, emphasising that even if expressed, it does not create a hazard. EFSA stated: "Group 1 ARMGs contains antibiotic resistance genes which (a) are widely distributed among soil and enteric bacteria and (b) confer resistance to antibiotics which have no or only minor therapeutic relevance in human medicine and have only restricted use in defined areas of veterinary medicine … No restrictions are required with this class of marker genes either for field experimentation or for placing on the market." [9]

Horizontal Gene transfer

The applicant assessed the likelihood of horizontal transfer of these transgenes, along with consideration given to recombination with soil bacteria. They did not expect any gene transfer to occur, in line with results from previous studies[10] and stated that in

the unlikely case it happened, all the genes are under the control of plant-specific promoters so would not be expressed.

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[10]. 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.

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.

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 1–2% 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[11,12,13].

The trial will be conducted at four sites in England:

- Rothamsted Research’s experimental farm site in Harpenden, Hertfordshire;
- Rothamsted Research’s experimental farm site in Bury St Edmunds, Suffolk;
- The John Innes Centre’s Experimental Field station in Bawburgh, Norfolk;

- The National Institute of Agricultural Botany (NIAB) trial site in Cambridge, Cambridgeshire.

The maximum area for the proposed trial at each site will be 400m^2, including both GM and controls, spacing between plots and the pollen barrier. It will comprise 60 plots in total, with a maximum of 30 plots being *SP1* gene-edited GM lines. Each plot will be of 1 metre square area (except at the NIAB site, where each plot will be 1.75 metres square). There will be a 0.4 metre separation between each plot (but 0.8 metres at the NIAB site) and also around the trial's edge. The maximum number of GM plants released per trial site will be 7500, assuming 250 plants are sown in each of the 30 plots. This application is for a one-year trial with the planting of the first crop in Spring 2025. Harvesting is planned for August/September 2025

ACRE noted that the separation distance required to prevent hybridisation between different wheat varieties when certified seed is produced for marketing purposes is 2 metres. The application proposes to sow a 2-metre-wide wheat pollen barrier of non-GM Fielder wheat around the trial, which is in line with ACRE's previous advice.

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 one 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 trial will have a 20m isolation distance in which no cereals or grass species will be left to grow, other than those being trialled under separate GM releases. ACRE considered the request for other GM trials to be grown in the isolation distance when necessary, and concluded there was no increased risk to the environment from such trials. This is with the provision that both trials include their own pollen barrier and all material from the site(s) is handled as GM material during harvest and disposal. ACRE noted that any hybrids between cross-fertile species would not have been authorised under the GM consents and therefore seed from them could not be planted in following growing seasons without a further deliberate release consent application or variation. If seed is retained to be planted in future seasons, then methods to prevent cross-fertilisation should be used, e.g. bagging prior to flowering, selection of cultivars that flower at markedly different times, non-compatible species. ACRE were also minded to emphasise that only GM trials with a similar allowance for isolation distance overlap could be grown in such a way.

The applicant requested the option for the 2m pollen barrier to be included within the 20m isolation distance, rather than as well as, for one of the trial sites. ACRE's previous advice for trials of this scale (and some larger) was that the 20m isolation distance is the main control, as supported by published research[14], and the pollen barrier is additional protection as the barrier crop can sometimes grow poorly or

asynchronously. There was some discussion on ensuring ACRE offers consistent advice on trial applications, including an emphasis that the isolation distance would not be reduced below 20m, whether it includes the pollen barrier or not. Notably, all the sites for this trial are on experimental or research farms, within fenced GM trial fields, further distancing the field trial from any crop destined for the food or animal feed chain. As such, ACRE emphasised that at all sites 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, fungicides, nitrogen, sulphur and other fertilisers.

The sites will be monitored regularly: at least weekly during the trial and at least monthly for two years after the trial. For the post-trial monitoring period, the trial area will remain fallow to enable monitoring of volunteers. The soil will undergo shallow cultivation to encourage such volunteers, by lightly tilling down to 5 cm depth. The persistence of such volunteers from wheat in cultivated soil has been studied for a long time and is well-characterised[15,16,17]. ACRE were content with the monitoring methods set out by the applicant, noting that they follow that of numerous previous GM deliberate release trials for wheat.

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. Plant material and seeds may be harvested during the growing period 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.

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.

Items arising from public representations

No public representations were received.

Bibliography

1. Ling, Q., Broad, W., Trösch, R., Töpel, M., Demiral Sert, T., Lymperopoulos, P., Baldwin, A., and Jarvis, R.P. (2019). Ubiquitin-dependent chloroplast-associated protein degradation in plants. *Science* 363: eaav4467

2. Khatodia S, Bhatotia K, Passricha N, Khurana SM, Tuteja N. (2016). The CRISPR/Cas Genome-Editing Tool: Application in Improvement of Crops. *Frontiers in Plant Science* 19: 506. Frontiers | The CRISPR/Cas Genome-Editing Tool: Application in Improvement of Crops

3. Ling, Q., and Jarvis, P. (2015). Regulation of chloroplast protein import by the ubiquitin E3 ligase SP1 is important for stress tolerance in plants. *Current Biology* 25: 2527-2534

4. Ling, Q., Sadali, N. M., Soufi, Z., Zhou, Y., Huang, B., Zeng, Y., Rodriguez-Concepción, M. and Jarvis, R. P. (2021). The chloroplast-associated protein degradation pathway controls chromoplast development and fruit ripening in tomato. *Nature Plants* 7: 655-666

5. 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

6. 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

7. 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)

8. Cram, D., Kulkarni, M., Buchwaldt, M. et al. WheatCRISPR: a web-based guide RNA design tool for CRISPR/Cas9-mediated genome editing in wheat. *BMC Plant Biol* 19, 474 (2019). https://doi.org/10.1186/s12870-019-2097-z

9. Opinion of the Scientific Panel on Genetically Modified Organisms on the use of antibiotic resistance genes as marker genes in genetically modified plants, The EFSA Journal (2004) 48, 1-18

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

11. 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.

12. 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.

13. Willenborg, C.J., Brûlé-Babel, A.L. & Van Acker, R.C (2009). Low crop plant population densities promote pollen-mediated gene flow in spring wheat (Triticum aestivum L.). *Transgenic Res* 18, 841–854. https://doi.org/10.1007/s11248-009-9267-y

14. Kang, HG., Chung, OC., Bae, TW., Sun, HJ, Song, IJ, Park, KW, Lim, PO, Lee, J, Lee, YE, Song, PS and Lee, HY (2021). Pollen-mediated flow of bar gene in transgenic herbicide-resistant turf grass Zoysia japonica. *Plant Biotechnology Reports* 15: 241–250. https://doi.org/10.1007/s11816-021-00667-4

15. 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.

16. 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.

17. Olena Kalinina, Simon L. Zeller, Bernhard Schmid. (2015). Persistence of seeds, seedlings and plants, performance of transgenic wheat in weed communities in the field and effects on fallow weed diversity, Perspectives in Plant Ecology, Evolution and Systematics, Volume 17, Issue 6, 2015, Pages 421-433, ISSN 1433-8319, https://doi.org/10.1016/j.ppees.2015.09.005.