Department for Environment, Food and Rural Affairs

Application for consent to release genetically modified higher plants for non-marketing purposes

Part A1: Information required under Schedule 1 of the Genetically Modified Organisms (Deliberate Release) Regulations 2002 (as amended)

Part I General information

1. The name and address of the applicant and the name, qualifications and experience of the scientist and of every other person who will be responsible for planning and carrying out the release of the organisms and for the supervision, monitoring and safety of the release.

Applicants:

Name	Address	Qualifications and Experience
Principal Investigator	Department of Biology University of Oxford, South Parks Road, Oxford OX1 3RB	BSc, PhD, Professor of Plant Cell Biology, Group Leader. Over 25 years' experience on plastid biology: plastid protein import and control of plastid biogenesis.
Research Scientist	Department of Biology University of Oxford, South Parks Road, Oxford OX1 3RB	BSc Biology, PhD. Research Scientist with over 10 years' experience in plant molecular biology.

The Rothamsted Farms team, the John Innes Centre (JIC) Experimental Research (Church Farm) team, and the National Institute of Agricultural Botany (NIAB) Farms team will be contracted to carry out part of the trial and have extensive experience conducting GM field trials. The responsible people will be:

Name	Address	Qualifications and Experience
Head of Farms	Rothamsted Research, West	BSc Agriculture, 20+ years' experience in agriculture

	Common, Harpenden, AL5 2JQ	research and farm Management.
Rothamsted Farm and trials delivery teams (planting, harvest, cultivations, input applications)	Rothamsted Research, West Common, Harpenden, AL5 2JQ and Rothamsted Research, Brooms Barn, Higham, Bury St Edmunds, IP28 6NP	Various academic backgrounds throughout the team, all specific job training as required while employed by Rothamsted Research Farms.
Field Experimentation Manager	JIC Field Station, Church Farm, Bawburgh, Norwich NR9 3PY	35 years' experience in agricultural field trials with 5 years' direct experience growing GM trials at JIC.
Field Operations Manager	JIC Field Station, Church Farm, Bawburgh, Norwich NR9 3PY	BASIS Foundation certificate. 4 years' experience at JIC with involvement in GM trials.
Field Team	JIC Field Station, Church Farm, Bawburgh, Norwich NR9 3PY	14 years' experience at JIC with involvement in GM trials for 6 years.
Field Team Staff	JIC Field Station, Church Farm, Bawburgh, Norwich NR9 3PY	Various academic backgrounds throughout the team, all specific job training as required while employed by JIC.
Research lead in Crop Genetic Resources, NIAB GM trial leader	NIAB Park Farm, Villa Road, Histon, Cambridge CB24 9NZ	BA (Cantab.), PhD Crop Genetics, 33 years' experience in crop genetics and breeding at JIC, Syngenta Seeds and NIAB. Currently lead contact for NIAB GM potato trial on behalf of The Sainsbury Laboratory, under consent

		22/R29/01.
Members of NIAB farm and trials delivery teams (planting, harvest, cultivations, input applications)	NIAB Park Farm, Villa Road, Histon, Cambridge CB24 9NZ	Various academic backgrounds throughout the team, all specific job training as required while employed by NIAB.

2. The title of the project.

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

Part II Information relating to the parental or recipient plant

3. The full name of the plant -

(a) family name Poaceae

(b) genus Triticum

(c) species *Triticum aestivum*

(d) subspecies N/A

(e) cultivar/breeding line Fielder

(f) common name Common wheat; Spring wheat; Bread wheat

4. Information concerning -

(a) the reproduction of the plant:

(i) the mode or modes of reproduction,

Reproduction is sexual, leading to formation of seeds. Wheat is approximately 99% autogamous under natural field conditions, with self-fertilization normally occurring before flowers open. Wheat pollen grains are relatively large and any that are released from the flower remain viable for between a few minutes and a few hours. Warm, dry, windy conditions may increase cross-pollination rates depending on the cultivar (see 6 below).

(ii) any specific factors affecting reproduction

Pollination, seed set, and grain filling are dependent on temperature, weather conditions, agronomic practice and pressure applied by pests and disease.

(iii) generation time; and

The generation time is 20-24 weeks; for Fielder (spring-wheat type), one season is normally from March/April to August/September.

(b) the sexual compatibility of the plant with other cultivated or wild plant species, including the distribution in Europe of the compatible species.

Wheat is naturally self-pollinating but under experimental conditions wheat can be crossed with various wild grasses. Of these, only the genera *Elymus* and *Elytrigia* (formerly *Agropyron*) are present in the UK but there are no reports of spontaneous hybrids between wheat and *Elymus/Elytrigia*. Wheat can also be forced using laboratory techniques to cross to rye, triticale and a limited number of other cereals.

5. Information concerning the survivability of the plant:

(a) its ability to form structures for survival or dormancy,

(b) any specific factors affecting survivability.

5 (a-b): Wheat is an annual species and survives from year to year only via seed production. In normal farming practice, mature seeds may fall from the plant prior to or at the time of harvest and not be collected. If not managed, these seeds may over-winter in the soil and germinate the following spring as “volunteers”. The cultivar Fielder is a US spring wheat which has no vernalisation requirement.

6. Information concerning the dissemination of the plant:

(a) the means and extent (such as an estimation of how viable pollen and/or seeds decline with distance where applicable) of dissemination; and
(b) any specific factors affecting dissemination.

6 (a-b): Pollen can be disseminated by the wind. Such dissemination is limited by the relatively large size and weight of wheat pollen. The risk of cross-pollination is also reduced by its short period of viability. Reports quantifying the rate of cross-pollination state that out-crossing rates are usually less than 1% (Hucl, 1996). Under certain growing conditions individual genotypes may have out-crossing rates of up to 4-5% (Griffin, 1987; Martin, 1990). Seed is usually retained by the plant until harvest, but a small proportion can be spilt to the ground at that time. Dispersal of seed prior to harvest by wind is unlikely, but possible by wildlife.

7. The geographical distribution of the plant in Europe.

Wheat is grown right across Europe and in temperate zones worldwide.

8. Where the application relates to a plant species which is not normally grown in Europe, a description of the natural habitat of the plant, including information on natural predators, parasites, competitors and symbionts.

Not applicable.

9. Any other potential interactions, relevant to the genetically modified organism, of the plant with organisms in the ecosystem where it is usually grown, or elsewhere, including information on toxic effects on humans, animals and other organisms.

Wheat plants have a range of pests and fungal pathogens. The main insect pests in the UK are three aphid (Homoptera: *Aphididae*) species, the bird cherry-oat aphid *Rhopalosiphum padi*; the grain aphid *Sitobion avenae*; and the rose grain aphid *Metopolophium dirhodum*; the orange wheat blossom midge *Sitodiplosis mosellana* (Diptera: *Cecidomyiidae*), and wheat bulb fly *Delia coarctata* (Diptera: *Anthomyiidae*). Wheat also interacts with beneficial insects, for example *Aphidius rhopalosiphi* (Hymenoptera: *Aphidiinae*) which attack aphid pests.

Wheat is not toxic and a major world bulk commodity food but may cause gastro-intestinal intolerance, coeliac disease and/or “bakers’ asthma” in susceptible individuals.

The plant lines intended for this multisite trial and the seeds arising from it will not enter the food or feed chains.

Part III Information relating to the genetic modification

10. A description of the methods used for the genetic modification.

Transgenic wheat plants were produced using the *Agrobacterium tumefaciens*-mediated transformation method described in Hayta *et al.*, 2019 and 2021. The constructs were introduced into immature embryos of *T. aestivum* cv. Fielder by *Agrobacterium*-mediated inoculation. Whole plants were regenerated and selected from immature embryos induced in tissue culture.

The introduced construct is a plasmid vector for inducing gene-targeted knockout mutants via the CRISPR/Cas9 system. This plasmid vector was assembled according to Smedley *et al.*, 2021 by using Golden Gate (GG, Type IIS restriction enzyme) modular cloning (MoClo) assembly. For targeting the three homoeolog copies of the gene of interest within each subgenome (A, B and D), two single guide RNA (sgRNA) sequences were selected for an area of homology in the first exon of the gene of interest for the three subgenomes (Guide_2 and Guide_3). The WheatCRISPR tool was used for the selection of the sgRNAs, as described in Smedley *et al.*, 2021. The predicted on-target and off-target cutting efficiencies were considered; the sgRNAs off-target score was predicted to have the value of “0”, equivalent to “no predicted hits” in other genic or intergenic regions according to the WheatCRISPR tool.

11. The nature and source of the vector used.

The vector used is the “L2_SP1_guides_2,3” final Level 2 binary vector (see vector map below) which derives from the assembly of “Level 1 plasmids” into the pGGG-M Level 2 acceptor plasmid (Smedley et al., 2021). The “L2_SP1_guides_2,3” binary vector contains the plant selection resistance gene for hygromycin (*hptII*), the Cas9 and the two sgRNA specific for the gene of interest.

All the Level 1 plasmids used for the assembly contain expression cassettes, compatible with MoClo assembly system (Smedley *et al.*, 2021), and are: OsActinP:*hpt*-int:35sT; ZmUbiP:Cas9:NosT; L1P4_SP1_g2; L1P5_SP1_g3. The L1P4_SP1_g2 and L1P5_SP1_g3 plasmids were generated by cloning the sgRNAs, Guide_2 and Guide_3 into the plasmids Level 1 P4_TaU6 and Level 1 P5_TaU6, respectively.

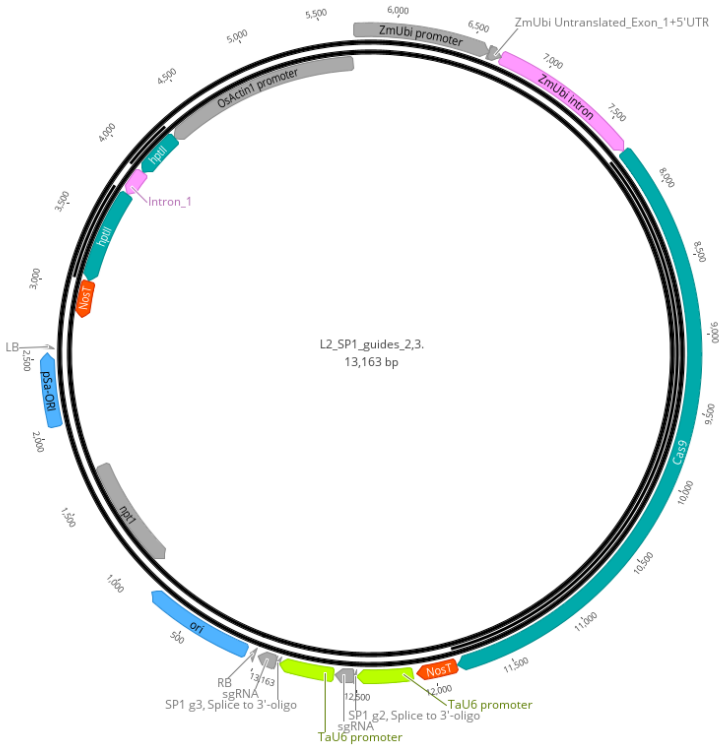

Figure. Level 2 binary vector “L2_SP1_guides_2,3”.

12. The size, intended function and name of the donor organism or organisms of each constituent fragment of the region intended for insertion.

The “L2_SP1_guides_2,3” region intended for insertion into the plant genome is described in the table below.

Element	**Function**	**Size (base pairs)**	**Donor organism**
LB	T-DNA left border	24	*Agrobacterium tumefaciens*

OsActin1 promoter	Promoter sequence	1417	*Oryza sativa*
hptII with intron	hygromycin phosphotransferase (or *hph*) selection gene containing the CAT1 intron from *Ricinus communis* catalase-1 gene	1216	*Escherichia coli* and *Ricinus communis*
NosT	Nopaline synthase terminator	263	*Agrobacterium tumefaciens*
ZmUbi promoter, 5'UTR and intron	Maize ubiquitin promoter with untranslated region and intron for driving constitutive expression	1988	*Zea mays*
Cas9	Endonuclease gene for the CRISPR/Cas9 system, generates RNA-guided double strand breaks in DNA	4140	*Streptococcus pyogenes*
NosT	Nopaline synthase terminator	263	*Agrobacterium tumefaciens*
TaU6 promoter	U6 promoter sequence	362	*Triticum aestivum*
SP1_g2	SP1_guide 2	21	*Triticum aestivum*
sgRNA	Guide RNA scaffold for the CRISPR/Cas9 system	125	*Streptococcus pyogenes*
TaU6 promoter	U6 promoter sequence	361	*Triticum aestivum*
SP1_g3	SP1_guide 3	21	*Triticum aestivum*
sgRNA	Guide RNA scaffold for the CRISPR/Cas9 system	125	*Streptococcus pyogenes*
RB	T-DNA right border	25	*Agrobacterium tumefaciens*

Part IV Information relating to the genetically modified plant

13. A description of the trait or traits and characteristics of the genetically modified plant which have been introduced or modified.

The CHLORAD (chloroplast-associated protein degradation) system is a “master

regulator" of plastid protein import; this is, of the mechanism whereby thousands of proteins that make up the chloroplasts are assembled (Ling *et al.*, 2019). SP1 and SP2 are key components of this system; mutations in these proteins directly affect the formation and operation of chloroplasts (Ling and Jarvis, 2015; Ling *et al.*, 2021).

CHLORAD is responsive to environmental and developmental cues; its manipulation *via* SP1 or SP2 can lead to traits that are of interest in the development of new, improved crop varieties; notably, delayed leaf senescence causing a functional "stay-green" phenotype associated with prolonged photosynthetic activity, and potentially improving yield. Functional stay-green is a valuable trait for improving tolerance of crops to stresses, such as salinity and drought; in addition, it is associated with disease resistance, e.g. spot blotch with causes major yield losses to wheat crops worldwide.

The genetically modified (GM) plant lines for these field trials are gene-edited wheat plants (cv. Fielder) with null levels of SP1. To generate *sp1* null mutants, the target gene was edited using the CRISPR/Cas9 system. The production of the edited plants initially required the introduction of a binary vector carrying a transgenic cassette necessary for the CRISPR/Cas9-mediated edition and for plant selection. This cassette was not segregated from the progeny of most of the selected lines; thus, the *sp1* gene-edited plant lines for these trials are GM plants because they contain the transgenic cassette inserted in a stable manner in their genome (denoted as GM+ in the table below), except for one line in which the transgenic cassette has been segregated (denoted as GM-).

The intended lines for this multisite trial are as described in the table below. The genetic screening identified (multi-) homeologue (single, double and triple) mutants for *SP1*; thus, their name describes their mutant genotype. The genetic editions per subgenome (A, B, and D) are described below in 14. The progeny of the lines has been genotyped to confirm (i) homozygosity of the genetic edition(s) per subgenome, and (ii) that further editing was not occurring in the subsequent generation in other homoeologue(s).

Edited plant line	**GM**	**Generation**
sp1-a5	+	T5
sp1-d1	+	T4
sp1-a6b5	+	T5
sp1-a2d1	+	T4
sp1-a5d1	+	T5
sp1-b4d1	-	T4

sp1-a2b1d1	+	T5
sp1-a6b5d2	+	T5
sp1-a7b5d7	+	T5

14. The following information on the sequences actually inserted or deleted:

(a) the size and structure of the insert and methods used for its characterisation, including information on any parts of the vector introduced into the genetically modified plant or any carrier or foreign DNA remaining in the genetically modified plant,

The size of the transgenic cassette inserted into the plant genome of the genetically modified plants is 10,641 base pairs. The cassette spans from the LB to the RB sequence (T-DNA Left- and Right-Border, respectively) and contains all the elements necessary for the CRISPR/Cas9 system to generate edits in the gene target, as well as the hygromycin selection marker, as depicted in the plasmid figure and detailed in the table above.

To identify the insertion of the transgenic cassette in the plant genome, PCR followed by gel electrophoresis was performed to determine the presence of the amplicon of the hygromycin marker-gene, as indicated below in Part IVA 24. Results showed that all the selected gene-edited lines (except for one) intended for this multisite trial carry the transgenic cassette. See details above in 13 (*i.e.*, GM +/-).

(b) the size and function of the deleted region or regions,

Commercially confidential information.

(c) the copy number of the insert, and

The copy number of the insert (*i.e.*, transgenic cassette) was not determined. However, the transgenic elements present in the insert are not expected to be a risk for the environment (see more details in Part IVA).

(d) the location or locations of the insert or inserts in the plant cells (whether it is integrated in the chromosome, chloroplasts, mitochondria, or maintained in a non-integrated form) and the methods for its determination.

The location(s) of the insert(s) of the GM lines was not determined but *Agrobacterium*-mediated transformation generates insertions in the plant nuclear genome (Faure, 2021; Gelvin and Kim, 2007; Tinland, 1996). In stable transformations the T-DNA, integrated into the plant genome, is transmitted along cell divisions to lead to a stable transformed plant (Faure, 2021). The hygromycin-resistance marker, as indicator of the GM trait, was identified across generations of

the lines intended for these trials; we therefore conclude that the location of the insert(s) is in the nuclear genome.

15. The following information on the expression of the insert

a). The genetic stability of the insert and phenotypic stability of the genetically modified plant.

The presence of the T-DNA insert was confirmed by genotyping for the hygromycin marker-gene in the progeny of the selected GM lines [as indicated in Section 14(a)]. In addition, the progeny of these lines shows stable edits induced by CRISPR/Cas9; this is, no further edits were found in other *SP1* homeologue(s) despite the presence of the gene-editing components of the transgenic cassette (see section 13 above).

Our preliminary observations of the GM lines growing in our greenhouses indicate there is no phenotypic variation among multiple individuals of each GM line selected for this multisite trial, and when compared to the control lines, except for the delayed senescence. Preliminary observations on morphology, flowering time, pollination and number of tillers of plants currently growing in the greenhouse also confirm there is no variation among individuals (8 to 10) per line or when compared to the non-GM relevant controls. Measurements of leaf chlorophyll content have not shown any significant variation in the chlorophyll concentration among individuals (6 to 8) per line or when compared to the non-GM controls. Overall, these preliminary results suggest the selected GM lines grow consistently.

b). Conclusions on the molecular characterisation of the genetically modified plant.

The plant lines selected for this multisite trial have been characterised at the molecular level: sequencing results of specific PCR amplicons have confirmed the presence of the transgenic cassette and the gene-edits in the target gene homeologue(s).

Part IVA Information on specific areas of risk

16. Any change to the persistence or invasiveness of the genetically modified plant and its ability to transfer genetic material to sexually compatible relatives and the adverse environmental effects arising,

The transgenic cassette is not anticipated to confer any advantage compared to conventional wheat cultivars with respect to persistence in agricultural habitats or invasiveness in natural habitats and no emergent hazard is predicted. The altered trait in the edited plants (*i.e.*, the delayed senescence leading to a functional stay-green phenotype) may improve the tolerance to biotic and abiotic stresses (such as drought, salinity, or foliar fungal diseases) besides yield (Danful *et al*., 2019; Kamal, *et al*., 2019); but an assessment of this potential tolerance improvement is not part of the field trials.

Although the GM lines are resistance to hygromycin, plants remain sensitive to all other herbicides. Also, it is not expected that the GM lines will differ from conventional wheat in their capacity to self- or cross-pollinate via sexual reproduction (see Sections 4 and 6).

The frequency of pollen-mediated gene flow (PMGF) in wheat is low even for plants in close proximity and decreases rapidly with distance to the pollen source; some studies have concluded that, at a distance of 10 m from the pollen source, PMGF events were reported under 0.5% for spring and winter wheat, even for farm-scale fields (see Foetzki *et al.*, 2012); thus, only a low rate of cross-pollination with closely adjacent wheat plants within the trial plots could potentially occur. In this multisite trial, management procedures to minimise the dissemination of pollen or seeds (*i.e.*, the pollen barrier surrounding the plots, and the 20 m with of fallow area) will further reduce the probability of cross-pollination outside the trial areas. Enclosing each trial site with a fence (with lockable double gates) to prevent the entry small and large mammals including unauthorised humans.

17. Any change in the ability of the genetically modified plant to transfer genetic material to microorganisms and the adverse environmental effects arising,

The transgene is integrated into the plant genomic DNA. It is not expected any change in the ability of the GM plants to transfer any genetic material to microorganisms; this reasoning is backed up by previous studies of horizontal gene transfer from plants to bacteria, suggesting that these events are extremely rare (Keese, 2008). Furthermore, all the transgenic elements (*i.e.*, the hygromycin selection marker, the Cas9 coding sequence and SP1-specific sgRNAs) are under the control of plant-specific promoters, as functional transcription units; thus, in the unlikely event of horizontal gene transfer the expression of the transgenes in soil microorganisms would be improbable unless they have been transferred as functional transcription unit.

18. The mechanism of interaction between the genetically modified plant and target organisms, if applicable, and the adverse environmental effects arising,

Not applicable; there are no target organisms.

19. Potential changes in the interactions of the genetically modified plant with non-target organisms resulting from the genetic modification and the adverse environmental effects arising,

The only potential change in the interactions of the GM plants with non-target organisms would be that the plants could cope better against fungal diseases but because of the genetic edition rather than the presence of the transgenic cassette. As mentioned above (Section 13), the stay-green trait can improve to some extent the resistance to diseases in the SP1 null mutants (e.g. spot blotch). However, if this

interaction changed it would not represent an adverse environmental hazard because the trials site will have measures to prevent dissemination of pollen and seeds.

On the other hand, the transgenic elements integrated in the plant genomic DNA are unlikely to be transferred to other organisms by horizontal gene transfer.

20. Potential changes in agricultural practices and management of the genetically modified plant resulting from the genetic modification, if applicable, and the adverse environmental effects arising,

Not known and not expected.

21. Potential interactions with the abiotic environment and the adverse environmental effects arising,

Not known and not expected.

22. Any toxic, allergenic or other harmful effects on human health arising from the genetic modification,

Preliminary assessments of wheat SP1 knockdown plants (with reduced SP1 activity) have shown that the stay-green phenotype is linked to significant increases in grain yield. The toxic, allergenic or other harmful effects on human health were not evaluated in the harvested grains but no undesired effects were expected in the quality of grains.

In the GM lines intended for this trial, the transgenic elements are not known to be pathogenic or allergenic to humans, and none of these DNA sequences are expected to result in the synthesis of products that are harmful to humans, other organisms or the environment.

All the transgenic elements (*i.e.*, plant-specific promoters and coding sequences) are unlikely to drive expression or to produce a product in a non-plant cell environment. In addition, although the gene-editing components were not tested for expression, the fact that no further edits were found in the progeny of each selected line despite the presence of the transgenic cassette, strongly suggests that these components are no longer expressed or are not active in later generations (Sections 13 and 15). Moreover, the sgRNAs are designed to target a gene that localises in the chloroplasts of plants, and importantly, no off-target sequences are predicted for this pair of sgRNAs.

Besides, the hygromycin resistance marker, present in the GM lines, is not expected to cause any harmful effects on human health. This antibiotic is not utilised in human clinical medicine. This resistance-marker gene is included in the “Group 1” of the ARMGs (antibiotic resistance marker genes), according to the EFSA (European Food Safety Authority), for which the EFSA scientific panel on GMs has indicated

that no restrictions are required in genetically modified plants, either for field experimentation or for placing on the market (Source: https://www.efsa.europa.eu/en/news/efsa-provides-scientific-advice-use-antibiotic-resistance-marker-genes).

In addition, because the modified plants will not enter the food or feed chains, we consider the potential toxicity or harmful effects to be negligible.

23. Conclusions on the specific areas of risk.

Although the copy number of the insert and the insertion site was not characterised in molecular detail, our data on: (i) the molecular characterisation, (ii) current phenotypic analysis, and (iii) the lack of potential off-target gene editing events, indicates that these GM lines are stable. In addition, this multisite trial will be of small scale per trial site (see Section 26), and these GM plants and harvested seeds will not enter the food or feed chains. Previous advice from ACRE for the release of GMs that contain these transgenic elements (*i.e.*, the Cas9 along with the sgRNA, and the selective marker) did not conclude that this created concerns with regards to risk assessing any environmental impact (Application Ref: 21/R08/01).

Overall, we conclude that the proposed field trials do not represent any risk to human or animal health, or to the environment including managed and unmanaged systems.

24. A description of detection and identification techniques for the genetically modified plant.

Identification by PCR and Sanger sequencing using specific primers. Specifically, PCR followed by gel electrophoresis analysis of the amplicon was performed to identify the presence of the transgenic cassette per individual plant, using specific primers for the *hptII* hygromycin-selection marker-gene.

For the detection of specific edits, PCR followed by Sanger sequencing analysis of the amplicon was performed. The primers used for this screening are designed to amplify individual target regions that span the target area, ~250-500 bp from the first and last sgRNA target sequence; the produced amplicons of size ~600-1,200 bp allowed the identification of small edits and large deletions (~50 to 430 bp).

25. Information about previous releases of the genetically modified plant, if applicable.

Not applicable.

Part V Information relating to the site of release

26. The location and size of the release site or sites.

The locations of the release sites will be:

(i) Rothamsted Research, West Common, Harpenden, Hertfordshire, AL5 2JQ. Grid-reference to be confirmed.

(ii) Rothamsted Research, Brooms Barn, Higham, Bury St Edmunds, IP28 6NP. Grid-reference to be confirmed.

(iii) JIC Church Farm, Bawburgh, Norwich, NR9 3PY. Grid-reference to be confirmed.

(iv) NIAB Park Farm, Histon, Cambridge, CB24 9NZ. Grid-reference to be confirmed.

The following information refers to all four locations, unless stated otherwise:

The trial area will comprise 60 plots, including a maximum of 30 plots planted with the *sp1* gene-edited GM lines (see Table in Section 13), as triplicates. The remaining plots will be planted with non-GM lines: *sp2* mutant lines (cv Cadenza); and cv Fielder and cv Cadenza as controls lines, all as triplicates. Note that the *sp2* lines are mutants for a second gene of interest that carry point mutations induced by chemical mutagenesis and that were selected by the TILLING method.

Each plot will have a surface of approximately 1 m^2 and will be separated from each other by approximately 0.4 m. At the NIAB Park Farm, the plots will have approximately a 1.75 m^2 surface, with a ~0.8 m interplot gap. The outer edge of the trial area will be surrounded by a 2 m barrier of non-GM wheat to function as a pollen barrier. The trial area, including spacing between plots and the pollen barrier, will cover a maximum of 400 m^2, of which a maximum of ~30 m^2 will be the GM plants growing surface (or ~52 m^2 at the NIAB Park Farm).

Except from the Church Farm trial site, during the release period, no cereals (other than those cultivated as part of this application or another GM trial) or grass species will grow in an area of at least 20 m width surrounding the perimeter of the pollen barrier and trial plots.

Due to the limited GM area in the Church Farm, during the release period in this trial site, no cereals (other than those cultivated as part of this application or another GM trial) will grow in an area of at least 20 m width surrounding the perimeter of the plot in which the GM plants are growing, and if this area is cropped, it is cropped with a non-cereal crop. This minimal 20 m distance to a GM plot may include the pollen barrier if there is not sufficient space to maintain the 20 m perimeter surrounding the pollen barrier and trial plots.

Also, in all four locations, the trials will be situated within the restricted area dedicated for use for GM experiments.

27. A description of the release site ecosystem, including climate, flora and fauna.

All the release sites are located in the East of England and are agricultural areas of

the experimental farms. In particular, the farms at Bury St Edmunds, Bawburgh, and Histon, pertain geographically to counties to the area denominated as East Anglia.

In general, the ecosystem of East Anglia hosts species of lowland grass and heath to semi-natural woodland, coastal marshes and freshwater reed beds. East Anglia has one third of the country's stock of the most productive Grade 1 and 2 soils, much of this from the area spreading from The Wash through The Fens into the southern parts of Suffolk. The water source in this region is the large chalk aquifer that stretches from the Northeast to the Southwest of the region (Source: https://www.3keel.com/wp-content/uploads/2018/01/healthy-ecosystems-east-anglia-lens.pdf).

On the other hand, Harpenden includes habitats such as acidic grasslands, woodlands, and the human-made Southdown Ponds. The acidic grasslands occur on soils with a pH of 5.5 or less and support an array of specialist species, a range of ground dwelling and burrowing insects. The Harpenden grassland hosts species such as grazing animals, bumblebees, green-winged orchids, harebells, and slender St John's wort. The Harpenden woodland is of secondary origin; the cessation of grazing has allowed tree seeds to germinate and grow on previous meadow areas to produce the largely oak-dominated woodland that occurs today. The Ponds are inhabited by waterfowl, amphibians and nesting birds (Sources: https://www.harpenden.gov.uk/green-spaces/harpenden-common, https://www.wildlifetrusts.org/habitats).

Specifically, the areas of the release sites, according to the MAGIC interactive map system from Defra (https://magic.defra.gov.uk/), include the habitats:

(i) Rothamsted Brooms Barn, Bury St Edmunds (IP28 6NP): arable and horticultural; acid, calcareous, neutral grassland, and broadleaved, mixed and yew woodland, as well as built-up areas and gardens. (ii) JIC Church Farm, Bawburgh (NR9 3PY): acid, calcareous, neutral grassland; bare ground, and dwarf shrub heath. (iii) NIAB Park Farm, Histon (CB24 9NZ): broadleaved, mixed and yew woodland; acid, calcareous, neutral grassland, and built-up areas and gardens. (iv) Rothamsted West Common, Harpenden (AL5 2JQ): acid, calcareous, neutral grassland; broadleaved, mixed and yew Woodland; and built-up areas and gardens.

In relation to climate, the temperature of the East of England shows both seasonal and diurnal variations; with a mean annual temperature of 9.5-10.5°C, the maximum temperatures are 6-8°C during winter and 20-23°C during summer. Through most of the region there are about 30 rain days (rainfall greater than 1 mm) in winter and less than 25 days in summer. Eastern England is one of the most sheltered parts of the UK to wind - the windiest areas are closer to the storms from the Atlantic (Source: https://www.metoffice.gov.uk/binaries/content/assets/metofficegovuk/pdf/weather/learn-about/weather/regional-climates/eastern-england_-climate-met-office.pdf).

28. Details of any sexually compatible wild relatives or cultivated plant species present at the release sites.

Wheat is a self-pollinating crop with very low rates of cross-pollination with other wheat plants. The only wild relatives of wheat commonly found across the UK are in the genera *Elymus* and *Elytrigia,* and there are no reports of cross-hybridisation between wheat and species of these genera. The two most common inland species are the common couch grass (*Elytrigia repens*, formerly *Agropyron repens*) and the bearded couch grass (*Elymus caninus*, formerly *Agropyron caninum*). Other related species, such as the sand couch (*Elytrigia juncea*, formerly *Agropyron junceum*), sea couch (*Elytrigia atherica*, formerly *Agropyron pycnanthum*) and hybrids are largely confined to coastal habitats.

According to the records of the National Biodiversity Network, in a 5 km radius of the respective postcodes of the trial sites (https://records.nbnatlas.org/explore/your-area), the incidence of the two most common grasses is as follows:

The common couch grass is prevalent in the areas around the JIC Church Farm (Bawburgh, NR9 3PY) and the Rothamsted West Common (Harpenden, AL5 2JQ) trial sites, but it less frequent around the Rothamsted Brooms Barn (Bury St Edmunds, IP28 6NP), followed by the NIAB Park Farm (Histon, CB24 9NZ). In general, the bearded couch is less common than the couch grass. The bearded couch is frequent around the West Common site and present to a lesser extent around the Brooms Barn site. This bearded couch grass has minimal number of records or no records at all around the Park Farm and the Church Farm, respectively.

The common couch grass propagates primarily by vegetative reproduction through rhizomes rather than by sexual reproduction – common couch is self-sterile and, as each spreading colony is usually a single clone, seeds are not often produced (Source: https://web.archive.org/web/20121007032740/http://apps.rhs.org.uk/advicesearch/Profile.aspx?pid=283). In the case of the bearded couch, a previous study using seeds from wild populations of this grass and of bread wheat (collected in the immediate vicinity of the bearded couch) in England, did not find any spontaneous hybridizations; this study concluded that introgression of bread wheat traits into the bearded couch population were improbable, disregarding the fact that these populations tend to grow in the same vicinity (Guadagnuolo, *et al.*, 2001).

In summary, the lack of reports of spontaneous hybrids between wheat and common couch or wheat and bearded couch, alleviates concerns in relation to potential cross-pollination events with GM pollen. Nevertheless, in all the trial sites, these grasses will be controlled along with other weeds in and around the trial site using standard farm practices. No wheat, cereals or grasses (including common and bearded couch) other than those cultivated as part of this application or another GM trial, will

be cultivated or allowed to grow within the trial site and the surrounding 20 m from the trials (or from a GM plot at the JIC Church Farm), as indicated in Section 26.

29. The proximity of the release sites to officially recognised biotopes or protected areas which may be affected.

The proximity of the release sites to protected areas, denoted as SSSI (Site of Special Scientific Interest) conservation designations, are:

(i) Bury St Edmunds (IP28 6NP): this release site is, on its northeast side, ~1.1 km away from the Breckland Farmland SSSI; to its west, the Newmarket Heath SSSI is located ~8.6 km away.

(ii) Bawburgh (NR9 3PY): to the northeast, by ~5.4 km, there is the Sweetbriar Road Meadows SSSI.

(iii) Histon (CB24 9NZ): to the south, by ~1.5 km, there is the Histon Road SSSI; and to its southwest there are the Traveller's Rest Pit and the Madingley Wood SSSIs, by ~2.7 km and ~4.6 km, respectively.

(iv) Harpenden (AL5 2JQ): to the northwest, by ~9 km, there is the Sherrardspark Wood SSSI.

Despite the proximity of the release sites to the above SSSIs, we consider that this multisite trial represents a minimal risk to any officially recognised biotopes to the protected areas because: (i) the trial sites are located in England within a sheltered area to wind (decreasing the chances of pollen dissemination), and (ii) the SSSIs are not in the immediate vicinity if the trial sites (all the trial sites are >1 km distant to the closest SSSI).

Part VI Information relating to the release

30. The purpose of the release of the genetically modified plant, including its initial use and any intention to use it as or in a product in the future.

The aim is to obtain proof-of-principle data for the use of CHLORAD as a technology for crop improvement.

To investigate the benefits of the stay-green phenotype observed in null mutants of *SP1* and *SP2* on wheat. Leaf-senescence time will be assessed *in planta* and yield-related traits (e.g., seed weight, size and number, starch content) will be assessed in the grains collected from the multisite field trials. The results would inform future efforts to develop transgene-free edited lines for crop improvement.

31. The foreseen date or dates and duration of the release.

The starting date will be Spring 2025. The seeds will be sown in March/April and harvested in August/September, finishing with all plants harvested and removed by Autumn 2025.

32. The method by which the genetically modified plants will be released.

In all the trial sites, seeds will be drilled using conventional plot-scale farm equipment.

33. The method for preparing and managing the release site, prior to, during and after the release, including cultivation practices and harvesting methods.

All four trial sites will be prepared by the staff of each farm, according to standard agronomic practices for wheat cultivation. If necessary, the ground preparation will include herbicide treatment and/or mechanical cultivation to clear the ground prior to drilling. The release in each trial site will be monitored regularly during all stages of development and harvested at maturity.

Harvest will occur during the months of August or September, once all the plants have senesced in full. The ears of the total number of plants per plot will be collected independently using a plot combine harvester. In all cases, each seed lot will be conditioned, threshed and stored appropriately in GM seed containers. The plot combine will be cleaned at the end of harvesting to ensure no grain is removed from the trial site. All the unwanted material will also be harvested and disposed by incineration or deep burial at a local authority-approved landfill site using an approved contractor. Transportation of waste material will be in secure containers.

34. The approximate number of genetically modified plants (or plants per square metre) to be released.

Considering a maximum of 30 GM plots per trial site and a maximum of 250 plants per plot: the maximum number of GM plants to be released per trial site is 7,500.

Part VII Information on control, monitoring, post-release and waste treatment plans

35-(1) A description of any precautions to maintain spatial and, as the case may be, temporal separation of the genetically modified plant from sexually compatible plant species.

(2) In sub-paragraph (1) “plant species” means-

(a) Wild and weedy relatives, or
(b) Crops

(a) See section 28 for information on wild relatives that are present in the area, noting that spontaneous crosses between these species and wheat have not been observed.

(b) Wheat is a self-pollinating crop with very low rates of cross-pollination with other wheat plants. Wheat can be forced, using laboratory techniques, to cross with rye, triticale and a limited number of other cereals, but spontaneous crossing in the field

is extremely rare if it occurs at all. Nevertheless, the outer edge of the trial has a 2 m wide strip of non-GM wheat to function as a pollen barrier.

In addition, in any of the trial sites, no wheat or other cereals or grasses (except for this application or another GM trial) will be cultivated or allowed to grow within 20 m of the trial; when necessary, herbicides will be used. At sowing, the drills will be filled on the trial area itself and will be thoroughly cleaned before leaving the trial area; all care will be taken to ensure that no seed remains on the surface. All the unwanted plant material will be treated according to standard operating procedures of the trial sites, for waste disposal of transgenic material. Finally, the grain obtained will be stored in appropriate seed storage facilities.

36. A description of the methods for post-release treatment of the site or sites.

The trial sites will receive standard farm practice as regard to herbicides, fungicides and fertilisers. The sites will be monitored regularly throughout the trial.

Following harvest, the areas of release will be lightly tilled to a depth of 5 cm to stimulate germination of any wheat plant volunteers. The release areas will be left fallow and monitored for wheat plant volunteers for 2 years following harvest. Any wheat plant volunteers detected in this period will be recorded before being destroyed prior to flowering, either by hand-pulling and autoclaving or by application of herbicides.

37. A description of the post-release treatment methods for the genetically modified plant material including wastes.

In all four trial sites, the ears of the total number of plants per plot will be collected independently using a plot combine; and the seeds will be conditioned and threshed. Seeds will be stored in appropriate GM seed containers. The GM and non-GM seed lots will be used for our research purpose (*i.e.*, to assess yield-related traits).

All the plants will be pulled from the ground and all straw and as many roots as possible. The pulled plants, roots and the rest of unwanted material will be placed in sealed, labelled bags or containers, and disposed by autoclaving. The waste treatment method will be in agreement with standard operating procedures for transgenic material in each trial site.

38. A description of monitoring plans and techniques.

The trial sites will be monitored regularly (on a weekly basis) for volunteers during the growing period and after the termination of the trial. The soil will be treated by lightly tilling down to 5 cm depth to encourage volunteers; and when detected, these volunteer plants will be recorded before being destroyed prior to flowering, either by hand-pulling and autoclaving or by application of herbicides.

39. A description of any emergency plans.

In the unlikely event of the integrity of any of the four trial sites being seriously compromised, the trial site(s) will be terminated and all plants (including GM, non-GM and control wheat plots, and pollen barrier rows) will be destroyed using a suitable herbicide or harvesting as deemed appropriate. All harvested material will be removed from the site and disposed of by incineration, autoclaving or deep burial at a local authority-approved landfill site using an approved contractor. Transportation of waste materials will be in secure containers. Site security staff and farm managers will be provided with the phone numbers of all key staff and with a standard operating procedure to follow.

40. Methods and procedures to protect the site.

The release site will be fenced to protect against animal damage and entry by unauthorised persons. Human access to the trial sites will be restricted to only those personnel who have been informed of the limitations and conditions of the consent. The release sites will be securely fenced. A sign will be posted indicating entry by unauthorised persons is prohibited.

The Rothamsted Research, JIC and NIAB trials teams have experience of previous GM field trials and the relevant management procedures at those sites. In addition, the Rothamsted Research has a movement-activated camera security system, and the trials team has a good working relationship with the local police, who will be informed of the trial and have experience of previous and current GM field trials at Rothamsted Research. GM inspectorate will have access to the trial sites on request.

Part VIII Information on methodology

41. A description of the methods used or a reference to standardised or internationally recognised methods used to compile the information required by this Schedule, and the name of the body or bodies responsible for carrying out the studies.

1. The selection of sgRNA target sequences for CRISPR/Cas9 and the construct assembly using Golden Gate MoClo assembly was performed by the Crop Transformation (Wheat) team at the JIC (Norwich Research Park) as described in Smedley *et al*., 2021. The WheatCRISPR tool, publicly available at https://crispr.bioinfo.nrc.ca/WheatCrispr/, was used for the selection of the sgRNAs, as described in Smedley *et al.*, 2021.

2. The procedures described by Hayta et. al. (2019, 2021), were used for the *Agrobacterium*-mediated transformation of immature embryos of the hexaploid spring wheat (cv. Fielder). These procedures were also followed for the selection, regeneration and rooting of transformants. The production of these transgenic plants was done by the Crop Transformation (Wheat) team at the JIC (Norwich Research Park).

3. The acclimatization of transformants, *i.e.*, the transplantation to soil, and further growth until harvesting the first generation of seeds (T1) was performed according to the procedure “How to grow wheat” (Source: https://www.wheat-training.com/introduction-to-wheat-growth/) by the scientist associated to the project at the Department of Biology, University of Oxford.

4. The screening for CRISPR/Cas9 genome edits was done by Sanger sequencing of PCR of subgenome-specific amplicons using gene-specific primers for each homoeolog among subgenomes. The presence of the *hptII*-Cas9-TaU6sgRNAs transgene cassette was identified by PCR using *hptII*-gene specific primers (*i.e.*, hygromycin resistance standard primers). The primer design, isolation of genomic DNA and screening for edits (*i.e.*, PCR and Sanger sequencing analysis) was performed according to standard molecular biology methods by the scientist associated to the project (Department of Biology, University of Oxford).

5. Further growth of T2-T5 generations, and bulk-up of seeds for the field trial was done under controlled environmental conditions by the scientist associated to the project at the Department of Biology, University of Oxford.

6. Further genotyping of T2-T5 generations was performed as above (step 4) by the scientist associated to the project at the Department of Biology, University of Oxford.

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