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.

The application is made on behalf of Rothamsted Research, at the following address:

Rothamsted Research,
West Common, Harpenden
Hertfordshire,
AL5 2JQ
UK

2. The title of the project.

Field assessment of ultra-low asparagine, low acrylamide, gene edited wheat.

Part II Information relating to the parental or recipient plant

3. The full name of the plant –

Subfamily: Pooideae

(a) family name	Poaceae; subfamily Pooideae
(b) genus	Triticum
(c) species	*Triticum aestivum*

(d) subspecies

(e) cultivar/breeding line Cadenza

(f) common name Common 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 heavy 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 on a variety to variety basis.

(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

For Cadenza sown as a winter-wheat type, one season is normally from September/October to August/September the following year. This variety can also be sown as a spring-wheat type, in which case the season is from March/April to August/September the same year.

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

Although wheat is naturally self-pollinating it can be crossed with various wild grasses under experimental conditions. Of these, only the genera *Elymus* and *Elytrigia* (formerly known as *Agropyron*) are present in the UK and there are no reports of spontaneous hybrids arising between wheat and species of these genera. Wheat can also be forced using laboratory techniques to cross with a limited number of cultivated cereals, including rye and triticale, but if crosses with these species do occur spontaneously in the field they must be extremely rare events.

5. Information concerning the survivability of the plant:

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

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'.

(b) any specific factors affecting survivability.

Cadenza is a UK milling variety, which is photoperiod-sensitive (ppd-D1) but has a negligible vernalising requirement and relatively high levels of frost tolerance, which means that it can be sown either as a spring or winter type under typical UK conditions (Whaley et al. 2004).

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.

Pollen can be disseminated by the wind but such dissemination is limited by the relatively large size and weight of wheat pollen. The risk of cross-pollination is also reduced by the relatively short period of wheat pollen viability. Reports quantifying the rate of cross-pollination state that out-crossing rates are usually less than 1 % (e.g. Hucl, 1996). However, 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.

(b) any specific factors affecting dissemination.

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 interact with a range of beneficial and pest insects and fungal pathogens. The main insect pests in the UK are the bird cherry-oat aphid (*Rhopalosiphum padi*), the grain aphid (*Sitobion avenae*) and the rose grain aphid (*Metopolophium dirhodum*), as well as the orange wheat blossom midge (*Sitodiplosis mosellana*) and the wheat bulb fly (*Delia coarctata*). Wheat also interacts with beneficial insects that attack aphid pests, such as *Aphidius rhopalosiphi*. Interactions with these insects are not expected to be affected in any way by the traits carried by the plants.

Wheat can become infected by several fungal pathogens in the UK, including *Septoria tritici* (*Mycosphaerella graminicola*), yellow rust (*Puccinia striiformis*) and brown rust (*Puccinia triticina*), as well as Fusarium head blight (*Fusarium graminearum*) and take-all disease (*Gaeumannomyces graminis* var. *tritici*). Good phytosanitary practice has been shown to be important in preventing the accumulation of high concentrations of free asparagine in the grain (Curtis et al., 2016), but the relationship between pathogen infection and asparagine metabolism requires further research if the mechanisms underpinning this observation are to be understood. Even so, we consider it extremely unlikely that reduced free asparagine concentration in the grain will make the wheat more resistant to fungal pathogens.

Wheat also interacts with multiple fungi, bacteria and protists in the rhizospere (Rossman et al., 2020), but these interactions are not expected to be affected in any way by the traits carried by the plants.

Wheat is not toxic and is a major world bulk commodity food, but it may cause gastro-intestinal intolerance, coeliac disease and/or ‘bakers’ asthma’ in susceptible individuals. This is not expected to be affected in any way by the traits carried by the plants in this trial.

Plants and seeds arising from this trial 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.

The aim of the study is to undertake a field trial of wheat cv. Cadenza in which one of the genes encoding asparagine synthetase, *TaASN2,* has been edited using the clustered, regularly interspaced, short palindromic repeats (CRISPR) system with the Cas9 nuclease. The production of the edited plants initially required the introduction

by genetic modification of a gene to express guide RNAs (gRNAs) that would interact with the Cas9 nuclease and target it to the *TaASN2* gene, as well as a gene to express the Cas9 nuclease itself, and a *bar* marker gene. These genes were carried on three separate plasmid vectors that were co-transformed into wheat cv. Cadenza by microprojectile bombardment.

The gRNAs were designed to target the first exon of all three homeologues of the *TaASN2* gene (i.e. *TaASN-A2*, *TaASN-B2* and *TaASN-D2*) but not the other asparagine synthetase genes (Raffan et al., 2021). The targeting sequences were attached to an optimised gRNA scaffold structure (Dang et al., 2015) to enable the gRNAs to interact with the Cas9 nuclease. The gRNAs, were: 1F (GGGGTGCGGCGACGAGTCGC), 2F (GGACTGGAGCGGCCTGCACC), 3R (GTAGAGCGGCTGGTCGCCGG) and 4R (CCTCGCAGTCACTGCCGGTC), with F and R denoting forward or reverse orientations. Their binding sites are shown in Figure 1A, along with the amino acid sequence encoded by the target region of the gene, with residues important for the activity of the enzyme highlighted.

The four gRNA-encoding DNAs were incorporated into a single, polycistronic gene, separated by tRNA sequences (Figure 1B), based on the system developed by Xie et al. (2015). This system exploits the endogenous tRNA-processing system to generate multiple gRNAs from a single transgene, enabling multiplex editing. The construct was assembled using a Golden Gate system (Engler et al., 2008) in plasmid pRRes209.481, downstream of a rice small nucleolar RNA U3 (*U3sno*) gene promoter to make pRRes209.481.ASN2. The successful generation of the plasmid was confirmed by nucleotide sequence analysis.

Plasmid pRRes209.481.ASN2 was introduced into scutella of wheat cv. Cadenza embryos by particle bombardment, together with pRRes.486, which carries a *Cas9* gene that has been codon-optimised for wheat, driven by a maize *Ubi1* promoter plus first intron, and a plasmid, pRRes1.111, containing the selectable marker gene (*bar*) encoding phosphinothrycin acetyl transferase (PAT), also under the control of a maize *Ubi1* gene promoter and first intron. Transformed embryos were recovered, cultured on selective medium and grown on to produce plants. These T0 plants were self-pollinated to generate T1 seed.

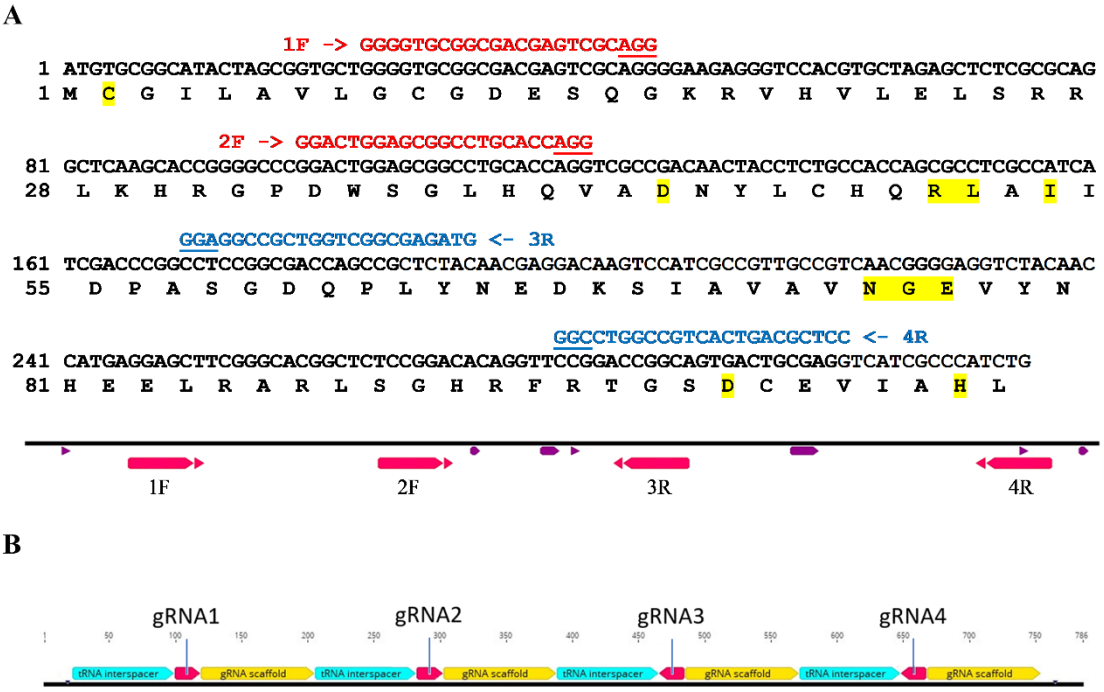


Figure 1.

A. Top: Nucleotide and derived amino acid sequence of the target region in the first exon of *TaASN2* showing the binding positions of the gRNAs. The forward gRNAs are shown in red and the reverse in blue, with PAM sequences (required for editing but not present in the gRNAs themselves) underlined. Residues known to be important in glutamine binding are highlighted in yellow. The nucleotide sequence shown is that of the A genome *TaASN2* gene, but the nucleotide sequences of the B and D genome *TaASN2* genes are identical at the gRNA binding sites. Bottom: schematic diagram showing the relative positions of the gRNA binding sites and the nucleotide sequences encoding key residues (purple). **B.** Diagrammatic representation of the polycistronic gene comprising the four gRNAs separated by tRNA sequences. From Raffan *et al.* (2021).

Seeds representing the T3 generation of plants produced in the transformation experiments have been sown to produce seed for the field trial, so the seed used for the trial will be the T4 generation.

11. The nature and source of the vector used.

The polycistronic gRNA gene under the control of a rice small nucleolar RNA U3 (*U3sno*) gene promoter was carried on plasmid pRRes209.481.ASN2. This was co-

transformed in wheat cv. Cadenza with plasmid pRRes.486, which carries a *Cas9* gene, codon-optimised for wheat, driven by a maize *Ubi1* promoter and first intron, and plasmid pRRes1.111, which carries the selectable marker gene (*bar*), which encodes phosphinothrycin acetyl transferase (PAT), also under the control of a maize *Ubi1* promoter and first intron. The plasmids were prepared in *Escherichia coli* DH5α competent cells (Genotype F- φ80lacZΔM15 Δ(lacZYA-argF)U169 recA1 endA1 hsdR17(rk-, mk+) phoAsupE44 thi-1 gyrA96 relA1 λ-), and purified using a Qiagen plasmid purification Midi kit.

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

Figure 2. Plasmid pRRes1.111. This plasmid contains the *bar* gene, encoding phosphinothricin N-acetyltransferase for tolerance phosphinothrycin-based herbicides, under the control of a maize *Ubi1* promoter and first intron.

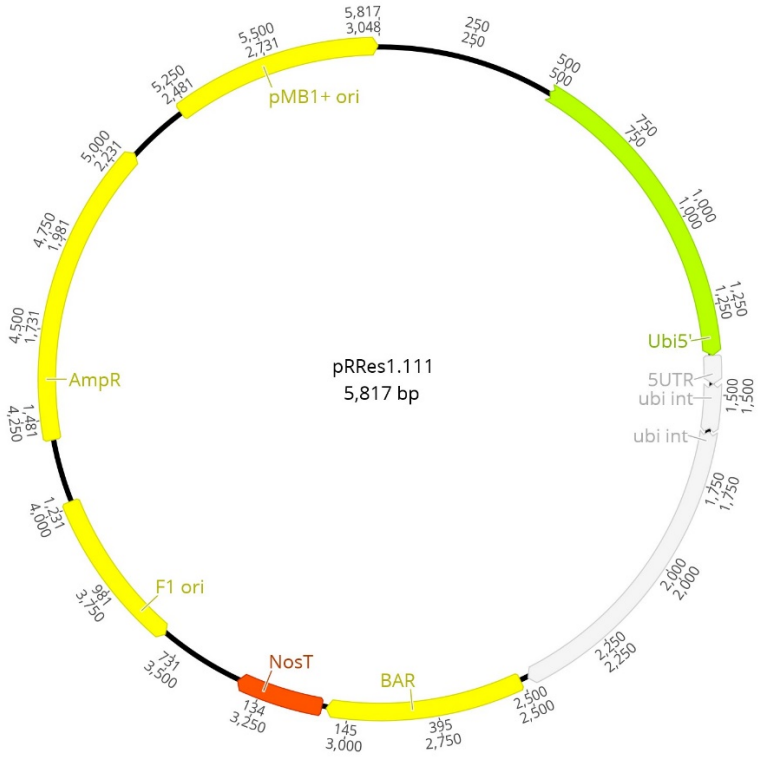


Table 1. Genetic elements in plasmid pRRes1.111

Element	Size	Donor Organism	Description and Intended Function
pMB1 ori	592bp	*E.coli*	Origin of replication for plasmid replication in *E. coli.*
F1 ori	459bp	*F1* bacteriophage	Origin of replication that allows for the replication and packaging of ssDNA.
AmpR	858bp	*E.coli*	Bacterial selection gene conferring resistance to ampicillin.
Ubi+intron	1988bp	*Zea mays*	Maize ubiquitin 1 (*Ubi1*) promoter + first intron driving constitutive expression in wheat.
Bar	554bp	*Streptomyces hygroscopicus*	Plant selectable marker gene encoding phosphinothricin acetyltransferase conferring resistance to herbicides based on phosphinothrycin.
nosT	246bp	*Agrobacterium tumefaciens*	Nopaline synthase gene terminator.

Figure 3. Plasmid pRRes217.486. This plasmid contains the *Cas9* gene, codon optimised for wheat, under the control of a maize *Ubi1* promoter and first intron.

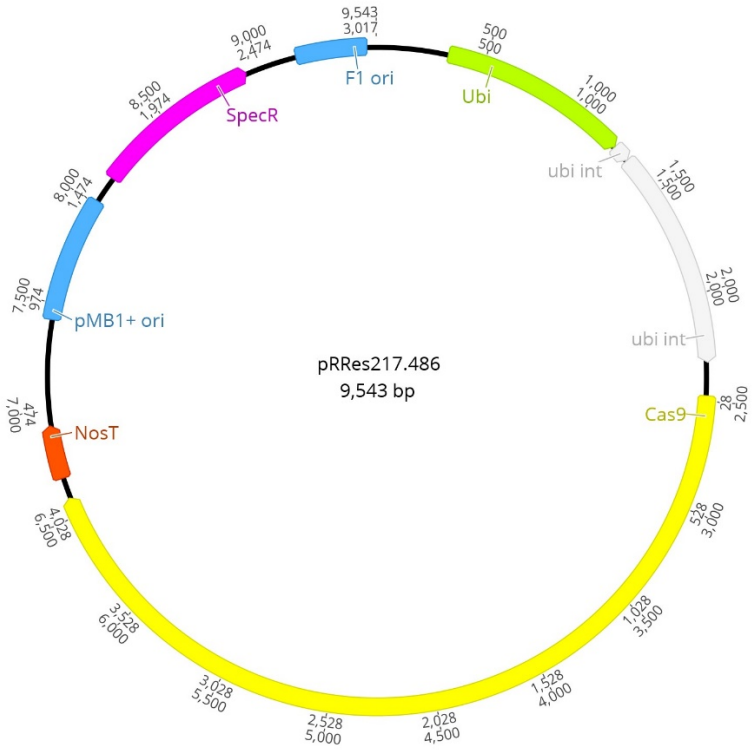


Table 2. Genetic elements in plasmid pRRes217.486.

Element	Size	Donor Organism	Description and Intended Function
pMB1+ ori	592bp	*E.coli*	Origin of replication for plasmid replication in *E.coli*.
SpecR	792bp	*E.coli*	Bacterial selection gene conferring resistance to spectinomycin.
F1 ori	333bp	*F1* bacteriophage	Origin of replication that allows for the replication and packaging of ssDNA.
Ubi+intron	1992bp	*Zea mays*	Maize ubiquitin 1 (*Ubi1*) promoter + first intron driving constitutive expression in wheat
Cas9	4108bp	*Streptococcus pyogenes*	Encodes the Cas9 RNA-guided DNA endonuclease enzyme, adapted for use in the CRISPR/Cas9 genome editing system and in this case codon-optimised for use in wheat.
nosT	244bp	*Agrobacterium tumefaciens*	Nopaline synthase gene terminator.

Figure 4. Plasmid pRRes482.ASN2. This plasmid contains the polycistronic gRNA gene, containing the 4 gRNAs, interspersed with tRNAs, under the control of a rice *U3sno* gene promotor.

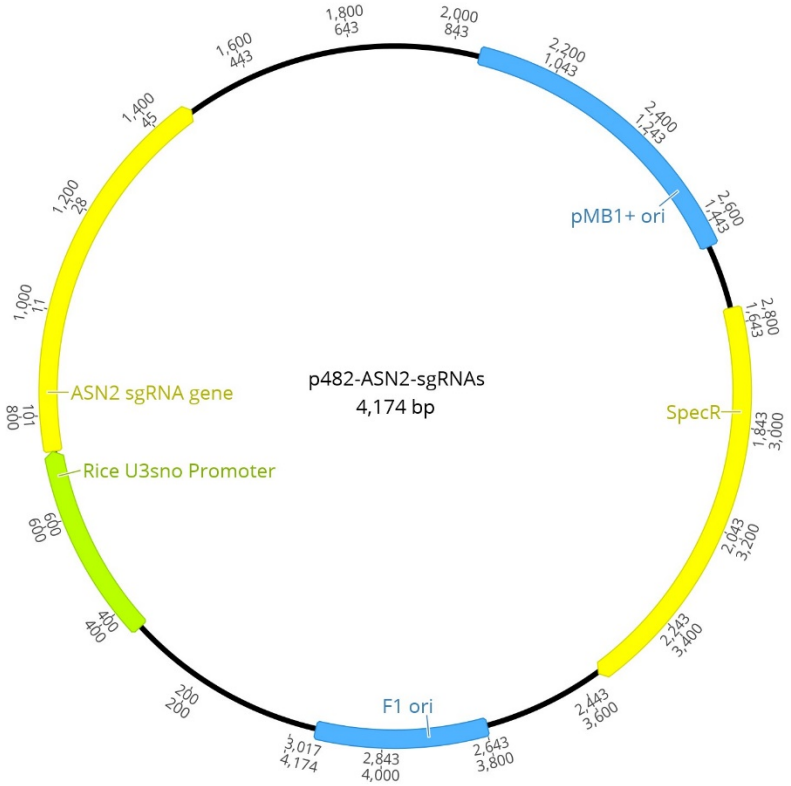


Table 3. Genetic elements in plasmid pRRes482.ASN2.

Element	Size	Donor Organism	Description and Intended Function
pMB1+ ori	592bp	*E.coli*	Origin of replication for plasmid replication in *E.coli.*
SpecR	792bp	*E.coli*	Bacterial selection gene conferring resistance to spectinomycin.
F1 ori	333bp	*F1* bacteriophage	Origin of replication that allows for the replication and packaging of ssDNA.
Rice *U3sno* gene promotor	380bp	*Oryza sativa*	Rice snoRNA U3 gene promotor driving constitutive expression in wheat.
TaASN2 sgRNAs	742bp	Synthetic	Polycistronic gRNA-containing gene consisting of gRNA target sequences, attached to a gRNA-scaffold and tandemly arrayed with tRNAs.

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 lines selected for the field trial are lines 23, 59 and 178 as described by Raffan et al. (2021). T3 seeds produced by selfing these plants have been sown to produce enough seed for the field trial, so the seed sown in the field trial will be the T4 generation.

Two plants of Line 23 were chosen to produce seed for the field trial: plants 23.60 and 23.75. The edits identified in the plants are shown in Figure 5. Plant 23.60 was homozygous for alleles A1, B1 and D1, while 23.75 was biallelic for alleles A1 and A2, biallelic for B1 and B2, and homozygous for D1.

Two plants were also chosen from line 59: 59.26 and 59.84. Plant 59.26 was homozygous for alleles A7, while 59.84 was biallelic for alleles A7 and A8 (Figure 5). Both plants were homozygous for alleles B5 and D4

A single plant was chosen for Line 178: plant 178.35. This plant was homozygous for allele A11 (Figure 5) and had wild-type alleles for the B and D genomes.

The proteins encoded by the edited alleles are all either extensively truncated or in the case of A7 lacks the key glutamine binding domain.

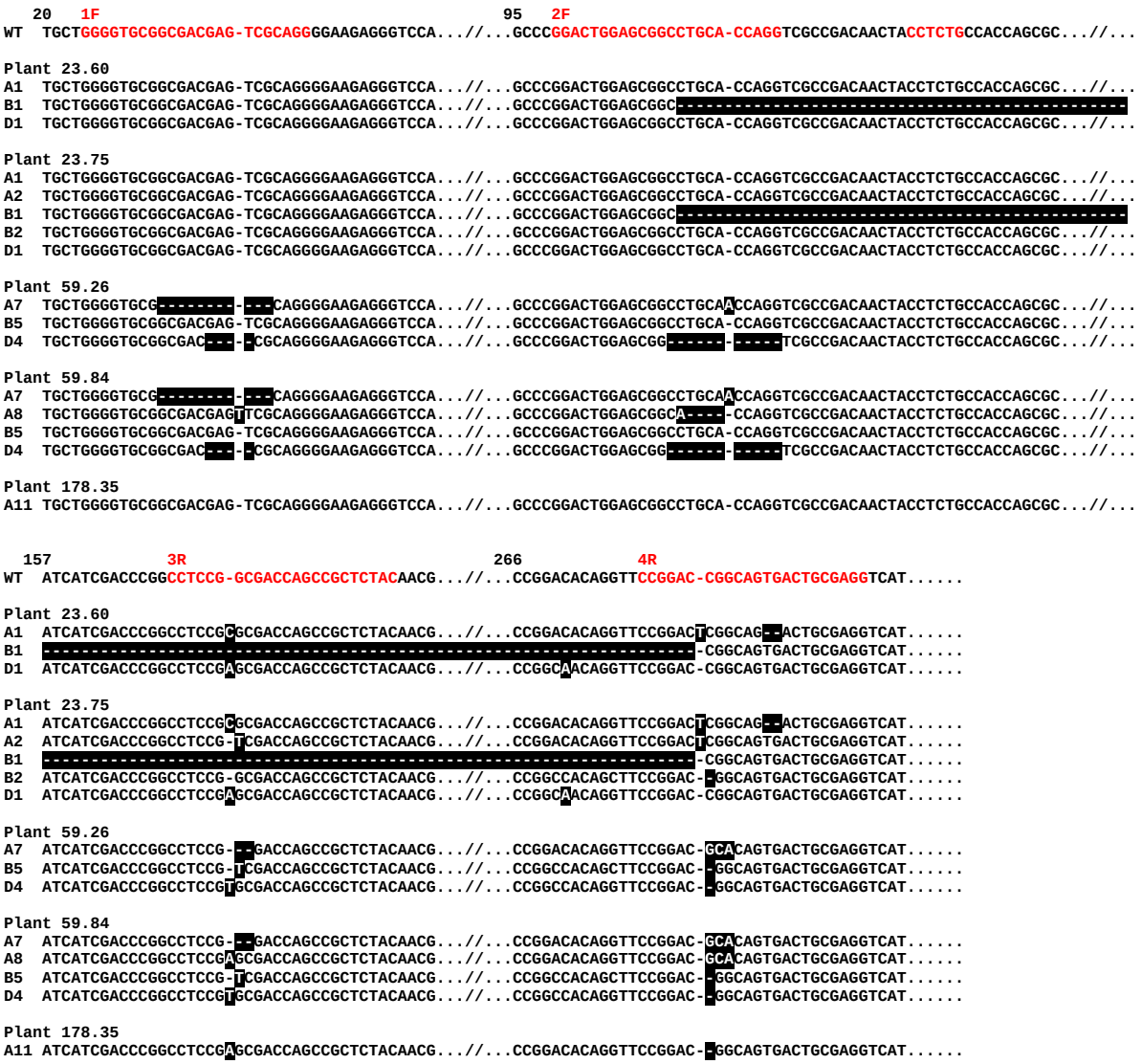


Figure 5.

Nucleotide sequences showing the edited *TaASN2* alleles present in the T2 generation of plants from which seed have been sown to grow plants to generate seed for the field trial. The A genome wildtype sequence is shown at the top, with the gRNA binding sites in red. Edits are highlighted in black. SNPs present in the wildtype sequences of the genes from genomes B and D are not highlighted. The different alleles present are numbered. Adapted from Raffan et al. (2021).

Wheat has five asparagine synthetase genes per genome, with *TaASN1*, *TaASN3.1*, *TaASN3.2* and *TaASN4* in addition to the targeted gene, *TaASN2*. *TaASN2* is the only one of the five to be expressed seed-specifically, with highest expression in the embryo (Gao et al., 2016; Curtis et al., 2019).

The major trait in the edited plants is low asparagine concentration in the grain (Raffan et al., 2021). The edited plants also show some changes in other free amino

acids, notably increases in the concentrations of free glutamine, glutamate and aspartate. Poor germination has also been observed for seed of lines 23 and 59, and grain weight was increased in lines 23 and 178 but not line 59.

In addition to these phenotypes, some of the plants still carry the *bar* gene encoding phosphinothrycin acetyl transferase and will tolerate phosphinothrycin-based herbicides.

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

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,

(b) the size and function of the deleted region or regions,
(c) the copy number of the insert, and
(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 transgenes are no longer required now that the *TaASN2* genes of the plants have been successfully edited. The process to remove them by segregation has begun, and the presence/absence of the *Cas9*, *bar* and *gRNA* genes in the plants being used to produce seed for the field trial is shown in Table 4. This has been assayed by polymerase chain reaction (PCR) using genomic DNA as template. The major aim of the field trial is to assess the performance of the plants under field conditions, but another aim is to enable enough plants to be screened to be able to detect some in which the transgenes have been lost altogether. We are applying for permission to run the field trial for 5 years and the transgenes are expected to have been eradicated by year 3.

Table 4. Results of genotyping (by PCR of genomic DNA) of plants being used to generate seed for the field trial, showing the number of plants out of a total of 20 in each case in which the *Cas9*, *bar* or gRNA transgenes have segregated away and are now absent.

Parent plant	***Cas9***	***bar***	***gRNA***
178.35	20	2	2
23.60	8	2	0
23.75	20	8	0
59.26	7	7	0
59.84	20	20	0

We have not analysed the copy number, position or structure of the remaining insertions nor sequenced the flanking genomic DNA.

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.

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

The *Cas9* and *bar* genes are under the transcriptional control of the maize *Ubi1* promoter + intron, while the gRNA gene is under the control of a rice U3sno gene promoter. These promoters give broadly constitutive expression in wheat.

With respect to editing, plants 178.35, 23.75 and 59.84 all lacked the *Cas9* gene and further editing cannot occur in subsequent generations derived from those plants. However, plants 23.60 and 59.26 did still contain a *Cas9* gene and this was segregating in the progeny of these plants being grown to produce seed for the field trial (Table 4). It is conceivable that editing could continue in plants in which the *Cas9* gene is still present.

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.

Plants in which the *bar* gene is still present will be resistant to control by herbicides based on phosphinothrycin (although other control measures will be unaffected). Reduced accumulation of free asparagine in the grain appears to have a negative effect on germination, and there is some evidence of increased seed size in 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. We therefore expect dissemination of pollen and seeds to be the same as for non-transgenic wheat plants, and the survivability of the plants in unmanaged systems to be reduced due to the germination effect.

There is no evidence of any change in the plants' ability to transfer genetic material to sexually compatible relatives.

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

The plasmids that were used for the transformation process possess a bacterial origin of replication and antibiotic resistance marker genes, and 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 (Table 4). 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, we estimate 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.

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.

As stated in Section 9, wheat plants have a range of pests and fungal pathogens. The main insect pests in the UK are the bird cherry-oat aphid (*Rhopalosiphum padi*), the grain aphid (*Sitobion avenae*) and the rose grain aphid (*Metopolophium dirhodum*), as well as the orange wheat blossom midge (*Sitodiplosis mosellana*) and

the wheat bulb fly (*Delia coarctata*). Wheat also interacts with beneficial insects that attack aphid pests, such as *Aphidius rhopalosiphi*. Interactions with these insects are not expected to be affected in any way by the traits carried by the plants.

Wheat can become infected by several fungal pathogens in the UK, including *Septoria tritici* (*Mycosphaerella graminicola*), yellow rust (*Puccinia striiformis*) and brown rust (*Puccinia triticina*), as well as Fusarium head blight (*Fusarium graminearum*) and take-all disease (*Gaeumannomyces graminis* var. *tritici*). Good phytosanitary practice has been shown to be important in preventing the accumulation of high concentrations of free asparagine in the grain (Curtis et al., 2016), but the relationship between pathogen infection and asparagine metabolism requires further research if the mechanisms underpinning this observation are to be understood. Even so, we consider it extremely unlikely that reduced free asparagine concentration in the grain will make the wheat more resistant to fungal pathogens.

Wheat also interacts with multiple fungi, bacteria and protists in the rhizospere (Rossman et al., 2020), but these interactions are not expected to be affected in any way by the traits carried by the plants.

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.

As stated above, plants still carrying the *bar* gene will be resistant to herbicides based on phosphinothrycin. Other herbicides will therefore have to be used to eradicate the plants. In addition, the seeds may need to be supplied with asparagine in order to germinate at the required rate. At glasshouse and field trial scale this can be applied over the soil after sowing, and methods more applicable to a farm setting (such as a seed coat) are still to be developed. No adverse environmental effects are predicted to arise from this.

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

None.

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

No toxic, allergenic or harmful effects on human health are envisaged. 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 (CONTAM Panel, 2015).

23. Conclusions on the specific areas of risk.

We conclude that the proposed field trial of these plants represents no risk to human or animal health, or the environment, including managed and unmanaged systems.

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

The genetically modified plants can be identified by PCR using primers specific for the *Cas9*, *gRNA* and *bar* genes. Primers have also been developed to detect the presence of the edits shown in Figure 5. This will become important when the plants are transgene-free.

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

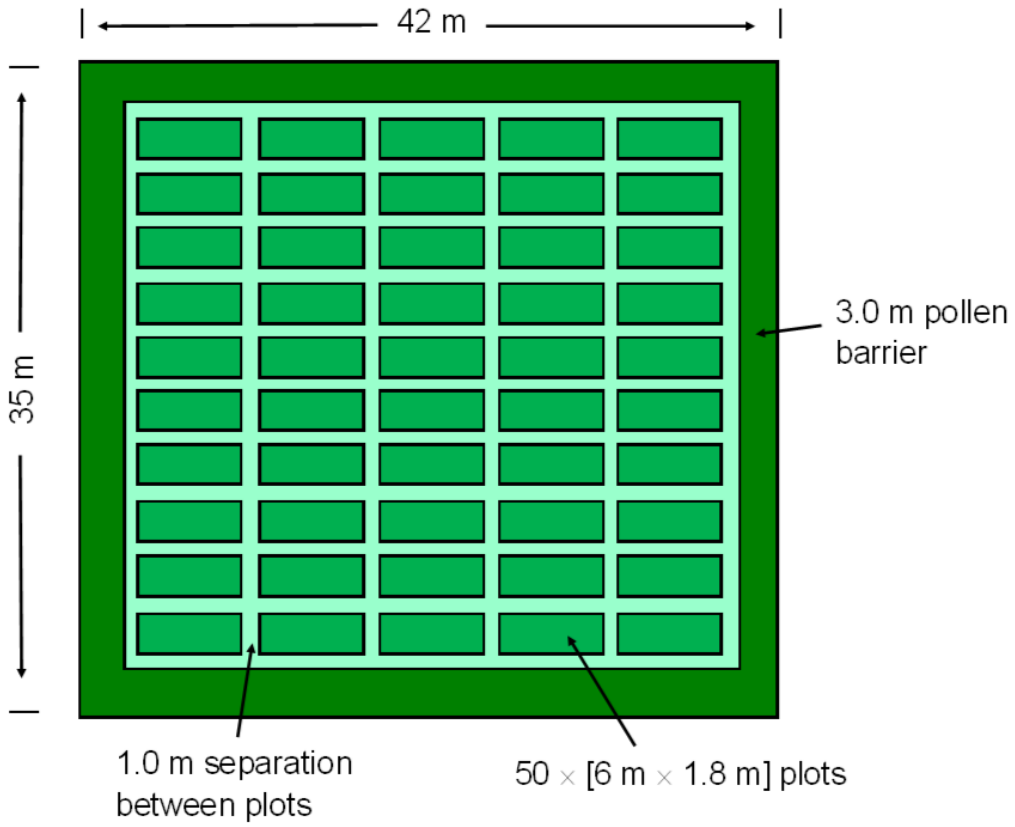
There have been no previous releases of these plants.

Part V Information relating to the site of release

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

The field trial will be sited within the GM field trial site at Rothamsted Research, Harpenden, UK, grid reference TL 1213. The area for the proposed field trial will be 42 m × 35 m (Figure 6). It will comprise 50 × [1.8 × 6 m] plots (Figure 6) planted with the five gene edited lines (178.35, 23.60, 23.75, 59.26 and 59.84), three non-GM lines of wheat cv. Claire in which the A genome *TaASN2* gene carries mutations induced by chemical mutagenesis, and wheat cv Claire and Cadenza controls. There will be five reps for each genotype. The separation between plots will be 1.0 m and there will be a wheat pollen barrier of 3 m which will completely surround the outer perimeter of the trial. No cereals or grass species will be cultivated or allowed to grow for a further 20 m from the outer edge of the pollen barrier. Enclosing the whole site will be a fence to prevent unauthorised access.

Figure 6. Plan of the proposed field trial.



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

The release site is an agricultural area forming part of an experimental farm. The flora and fauna are typical of agricultural land in the south-east of England.

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 in the UK are in the genera *Elymus* and *Elytrigia* (formerly known as *Agropyron*) and there are no reports of spontaneous cross-hybridisation between wheat and species of these genera. The two most common inland species are common couch (*Elytrigia repens* (= *Agropyron repens*)) and bearded couch (*Elymus caninus* (= *Agropyron caninum*)). Other related species, such as sand couch (*Elytrigia juncea* (= *Agropyron junceum*)), sea couch (*Elytrigia atherica* (= *Agropyron pycnanthum*)) and hybrids are largely confined to coastal habitats.

Common couch is quite widespread on the Rothamsted estate, whereas bearded couch is confined to woods and hedgerows. Common couch propagates primarily by vegetative reproduction (rhizomes), rather than by sexual reproduction, and no reports of wheat × couch spontaneous hybrids have been recorded. Nevertheless, common couch will be controlled along with other weeds in and around the trial site using standard farm practices. No wheat, other cereals or grasses, including common couch, will be cultivated or allowed to grow within 20 m of the trial.

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

There are no protected areas near the trial site.

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.

This is a research trial to investigate the effect of knocking out the *TaASN2* gene on free asparagine accumulation in wheat grain in the field. It will also 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. The formation of acrylamide in heated flour produced from the grain harvested from the trial will also be measured. The number of plants that can be grown in the field as opposed to a glasshouse will also facilitate the identification of plants in which the transgenes have segregated away. 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.

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

The trial will run for five seasons, from autumn 2021 to summer 2026. In each season, the plants will be sown in September/October and harvested in August/September the following year.

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

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.

The site will be prepared according to standard agronomic practices for winter wheat cultivation. 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. All other material, including that from the pollen barrier rows, will be harvested 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.

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

Twenty-five of the 50 plots will be planted with GM lines, adding up to 270 m2 (25 × [6 × 1.8 m]. Planting density will be approximately 300 seeds per m2, so the total number of GM plants in the trial each year will be approximately 81,000.

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 never 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 3 m-wide strip of non-GM wheat to function as a pollen barrier (Figure 6). In addition, no wheat, other cereals or grasses will be cultivated or allowed to grow within 20 m of the trial. 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. Phosphinothrycin-based herbicides will not be used in the trial, with alternatives such as glyphosate being used when/where necessary. Bird-scaring devices including gas guns and hawk kites will be used to keep birds out during the growing season.

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

The trial will receive standard farm practice as regard to herbicides (except that phosphinothrycin-based herbicides will not be used), fungicides, nitrogen, sulphur and other fertilisers. The site will be monitored regularly throughout the trial. The trial site will be moved within the Rothamsted GM field trial area from year to year to prevent the plants being affected by take-all disease (*Gaeumannomyces graminis* var. *tritici*) and to allow site monitoring.

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

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. Grain that is not required for analysis or to provide seed for future trials 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. 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.

Once the trial is concluded, or when the site is moved from one year to the next (Section 36), the trial area will remain in stubble for the following year to enable monitoring of volunteers and a broad spectrum herbicide such as glyphosate will be applied as required. Shallow cultivation will be used to encourage volunteers to germinate before treatment with the herbicide.

38. A description of monitoring plans and techniques.

The site will be monitored regularly (at least weekly) during the growing period (September/October to August/September) and for two years following the termination of the trial. Records will be kept of each visit.

39. A description of any emergency plans.

In the unlikely event of the integrity of the site being seriously compromised, the trial 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.

Rothamsted Research 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. The trial will be contained within the approximately 3 ha GM field trial site, which is protected by a fence and has a movement-activated camera security system.

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. Genetic transformation of wheat (*Triticum aestivum*) cv. Cadenza was performed by the Cereal Transformation Group, Rothamsted Research, using a PDS-1000/HeTM Biolistic Particle Delivery System (BioRad, Watford, UK), as described (Sparks and Doherty, 2020).

2. Characterisation of CRISPR-induced mutations (edits) in the GM plants was performed on DNA that was extracted from leaves using standard protocols (Raffan

et al., 2021). Next Generation Sequencing (NGS) analyses were used to identify and characterise edits in the *TaASN2* homeologues after amplification of the target region by PCR. NGS was performed using a MiSeq v2 Benchtop Sequencing System (Illumina, Cambridge, UK) at the Bristol Genomics Facility (University of Bristol). PANDAseq version 2.11 (Masella et al., 2012) was used to assemble the original fastq paired reads into contigs and demultiplexed by plant using a bespoke perl script. The fasta file for each plant was aligned to the reference sequence using a Bowtie 2 build version 2.3.4.1 (Langmead and Salzberg, 2012).

3. Amino acid analyses were performed on individual seeds from T1 plants (i.e. T2 seeds). Free amino acids were extracted using standard protocols and derivatised using an EZfaast Amino Acid Analysis Kit (Phenomenex, Macclesfield, UK). They were analysed using an Agilent 6890 GC-5975-MS system (Agilent, Santa Clara, CA) in electron impact mode, as described (Curtis et al., 2018; Elmore et al., 2005). Data were generated using the Agilent Chem Station app.

T3 grain were milled to wholemeal flour in a coffee grinder. Free amino acids were extracted as described (Curtis et al., 2018), derivatised with o-phthalaldehyde (OPA) and analysed by HPLC, as described by Noctor and Foyer (1998), using an Agilent 1100 HPLC system (Agilent Technologies, US) equipped with a Kinetexcolumn (2.6 µm XB-C18 100 Å LC Column, 150 x 4.6 mm; Phenomenex, UK) with an FLD detector (Agilent G1321D). Full details are provided by Raffan et al. (2021).

4. Statistical analyses were performed using GenStat (2018, 19th edition, © VSN International Ltd, Hemel Hempstead, UK).

5. The presence of the *Cas9*, *bar* and *gRNA* genes in the plants being grown to provide seed for the field trial was assessed by PCR using primers specific for the transgenes, with the plasmids used for transformation as positive controls.

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