Department for Environment, Food and Rural Affairs

Part A1: Application for consent to release genetically modified higher plants for non-marketing purposes

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

Part 1: General information

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

Applicant

Project Leader: Scientist with overall responsibility for the application. BSc, PhD, Professor, Group Leader. Over 25 years' experience in wheat genetics and wheat genomics. Lead applicant for previous releases 21/R52/01 and 19/R52/02.

Directorate, John Innes Centre, Norwich Research Park, Norwich, NR4 7UH

Scientists

Postdoctoral Scientist: Scientist (BSc, PhD) with 16 years' experience in wheat genetics and genomics and molecular biology.

Research Assistant: Scientist (BSc) who will be responsible for the day-to-day management and phenotyping of the field experiment. Over 26 years' experience in wheat genetics and wheat field experiments and scientist for previous releases 21/R52/01 and 19/R52/02.

Department of Crop Genetics, John Innes Centre, Norwich Research Park, Norwich, NR4 7UH

Field Experimentation Manager: Responsible for the husbandry of the wheat lines with 35+ years' experience in agricultural field trials with 7 years' direct experience growing GM trials at JIC.

Field Operations Manager: Assisting with field operations. BASIS Foundation certificate and 5 years' experience at JIC with involvement in GM trials.

JIC Field Station, Church Farm, Bawburgh, Norwich NR9 3PY

2. The title of the project

Improving grain yield in wheat by combining overexpression of the grain-specific expansin gene *TaExpA6*, driven by the Puroindoline-b (*PinB*) promoter, with loss-of-function mutations in *GW2* grain weight regulators or via loss-of-function mutations in the *GNI1* and *GNI2* floret fertility regulators.

Part 2: Information relating to the parental or recipient plant

3. The full name of the plant

a. family name: Poaceae

b. genus: *Triticum*

c. species: *aestivum*

d. subspecies: not applicable

e. cultivar or breeding line: Paragon; Skyfall; Bamford (for new transformants); Paragon BC4 (for crossed lines)

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 fruits (caryopsis) which are traditionally referred to as seeds or grains. 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 cultivar-to-cultivar basis (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

The generation time is 24 weeks for Paragon (spring-wheat type), normally from March/April to August in the UK. Both Bamford and Skyfall are classed as winter wheats and require several weeks of vernalisation (8 and 3 weeks, respectively). For these lines, one field season is normally from October to August (42 weeks) in the UK, although Skyfall can be planted in early Spring if weather and conditions permit.

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, and 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”.

6. Information concerning the dissemination of the plant

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

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% (e.g. 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 in temperate zones worldwide, mainly in Europe, North America, South America, and Asia.

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 wheat grain harvested from the trial is not intended for general human or animal consumption.

Part 3: Information relating to the genetic modification

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

Two parallel strategies are employed in this application:

Strategy 1 – Transgenic overexpression of *TaExpA6* driven by the *PinB* promoter, in a *gw2* mutant background (BC_4 lines):

An existing transgenic wheat event carrying a grain-specific overexpression construct of the wheat *TaExpA6* expansin gene (event pEW279-EXP; previously authorised under Chilean SAG Resolution Nº 2899/2018 and used in field trials at Universidad Austral de Chile) (Calderini *et al.*, 2021) was crossed into a Paragon background carrying EMS-induced loss-of-function alleles at the wheat *GRAIN WIDTH 2* (*GW2*) locus (*GW2-ABD* triple mutant alleles generated by ethyl methanesulphonate (EMS) mutagenesis) (Wang *et al.*, 2018). In the pEW279-EXP event, *TaExpA6* expression is driven by the endosperm-specific Puroindoline-b (*PinB*) promoter from wheat, which confers strong, grain-preferential expression. Recurrent backcrossing over four generations (BC_4) in a Paragon background was used to combine the *TaExpA6* transgene and selected *gw2* mutant combinations. BC_4 lines have been genotyped and selected to carry the following genotype classes: wild-type (WT), *TaExpA6* transgene only, *gw2-A* ± *TaExpA6*, *gw2-AD* ± *TaExpA6*, and *gw2-ABD* ± *TaExpA6*, all within a Paragon genetic background.

Strategy 2 – De novo transformation of UK elite cultivars with *PinB*::*TaExpA6* and CRISPR-mediated editing of *GW2* and *GNI1/GNI2*

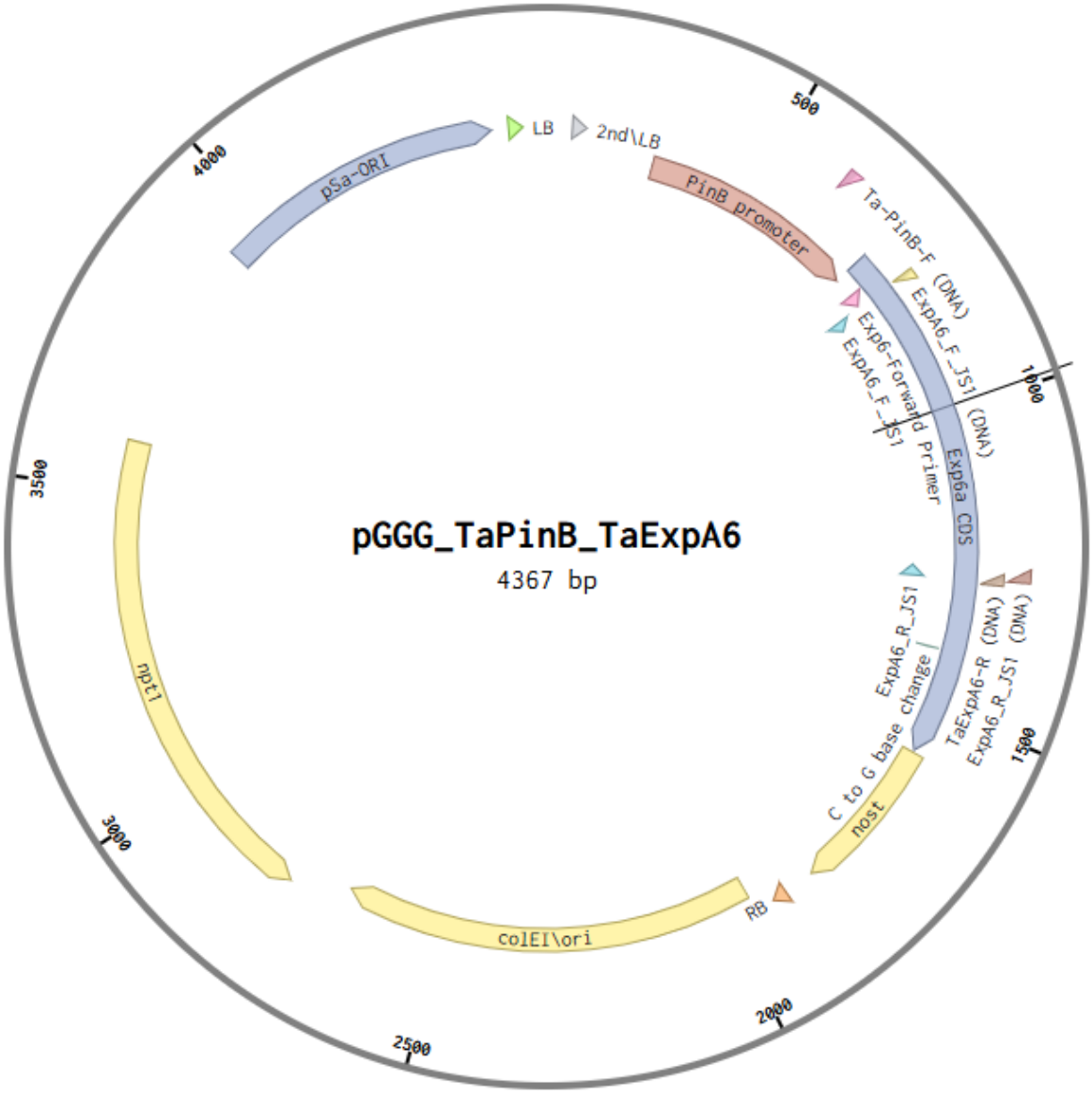
The *PinB*::*TaExpA6* construct used in Strategy 1 (endosperm-specific *PinB* promoter from wheat driving expression of the wheat *TaExpA6* expansin gene) has been independently re-introduced into three UK wheat cultivars (Paragon, Skyfall, and Bamford) by

Agrobacterium-mediated transformation of immature embryos, following the protocols of Hayta *et al.* (2019, 2021). This new *TaExpA6* construct does not contain the *hptI* hygromycin resistance gene; instead, transformants were identified by PCR screening using primers spanning the *PinB* promoter-*TaExpA6* junction (Ta-PinB-F / TaExpA6-R; see Section 24). Additionally, CRISPR/Cas9-mediated knockout constructs targeting all three *GW2* homoeologous alleles (A, B, and D subgenomes) have been introduced into the same cultivars; the CRISPR construct retains the *hptI* hygromycin resistance gene as a selectable marker. Selected transgenic and gene-edited lines have been advanced through glasshouse generations to achieve homozygosity.

11. The nature and source of the vector used

TaExpA6 overexpression construct (*PinB*::*TaExpA6*)

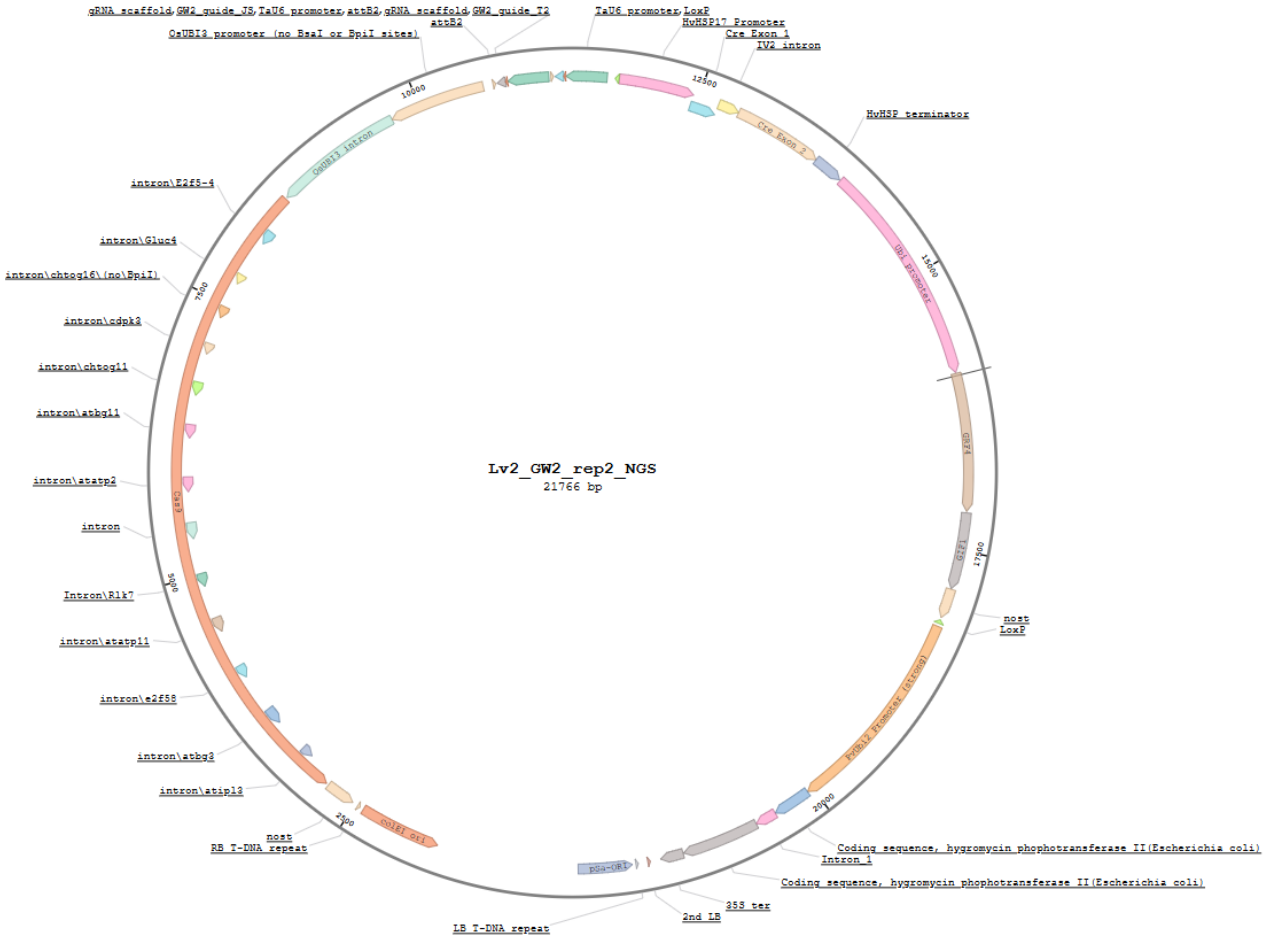
The *TaExpA6* overexpression construct (pEW279-EXP) and the reconstituted equivalent are based on a binary vector backbone. It contains the endosperm-specific *puroindoline-b* (*PinB*) promoter from wheat (*Triticum aestivum*) driving expression of the *TaExpA6* (*Triticum aestivum Expansin A6*) coding sequence, together with a transcriptional terminator. The *PinB* promoter is specifically active in the starchy endosperm of developing wheat grains and is not expressed in vegetative tissues. The reconstituted *TaExpA6* construct does not contain the *hptI* hygromycin resistance gene; it was used in transformation without a plant-selectable marker, with transgenic events identified by PCR. The construct was generated and characterised at John Innes Centre and is the same construct used in the Chilean SAG-authorised field trials (event pEW279-EXP; SAG Resolutions Nº 2833/2018 and 2899/2018).



CRISPR/Cas9 *GW2* editing construct

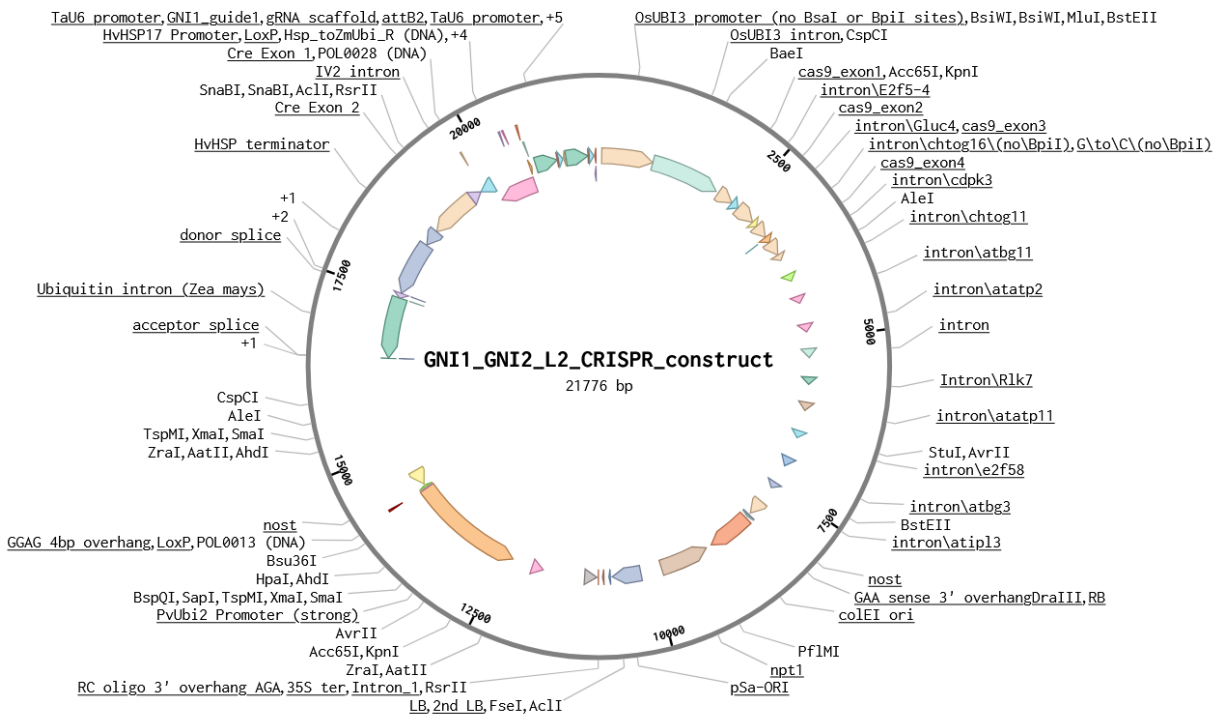
The CRISPR/Cas9 editing vector, designated pGGG (S1) Purple, is a binary vector assembled using Golden Gate (Type IIS restriction enzyme) MoClo assembly, as described in Smedley *et al.* (2026). The final vector contains: a *Zea mays*–codon-optimised *Cas9* containing 13 introns (Cas9-int) (Grützner *et al.*, 2021) under control of the rice *Ubiquitin (OsUbi)* promoter; two wheat *TaU6*-driven single guide RNA (sgRNA) sequences targeting conserved sequences in the first exon of the wheat (*Triticum aestivum*) *GW2* homoeologs across the A, B, and D subgenomes; maize *Ubiquitin 1* (*ZmUbi*) promoter driving a GRF4–GIF1 cassette (Debernadi *et al.* 2020); and the *hptI* hygromycin resistance gene under control of the switchgrass *Ubiquitin 2* (*PvUbi2*)

promoter as a plant selectable marker and detection marker. Guide RNA sequences were designed using the WheatCRISPR tool (Cram *et al.*, 2019).



CRISPR/Cas9 *GNI1/GNI2* editing construct

We used the pGGG (S1) purple binary vector, as described for *GW2*, to target the homoeologs of the wheat *GRAIN NUMBER INCREASE 1* (*GNI1*) (Sakuma et al., 2019) and *GRAIN NUMBER INCREASE 2* (*GNI2*) (Sakuma et al., 2026) genes; the only difference being in the single guide RNA (sgRNA) sequences. For this construct, two wheat *TaU6*-driven single guide RNA (sgRNA) sequences targeting conserved sequences in the second exon of *GNI1* and *GNI2* homoeologs across the A, B, and D subgenomes were used. Guide RNA sequences were designed using the WheatCRISPR tool (Cram *et al.*, 2019).



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

TaExpA6 overexpression construct (*PinB*::*TaExpA6*) – key elements

Element	Donor organism	Function
PinB promoter (Puroindoline-b promoter)	*Triticum aestivum*	Endosperm-specific promoter driving high-level expression of *TaExpA6* in the starchy endosperm of developing wheat grains; not expressed in vegetative tissues. REFSEQ v.1.1: *TraesCS7B02G431200*
TaExpA6 coding sequence	*Triticum aestivum*	Encodes *Expansin A6* protein; promotes cell wall loosening in developing grains, increasing grain size and weight. REFSEQ v.1.1: *TraesCS4A02G034200*
Transcriptional terminator (nosT or equivalent)	*Agrobacterium tumefaciens* or equivalent	Transcriptional terminator for the *TaExpA6* expression cassette

Element	Donor organism	Function
T-DNA left and right borders	*Agrobacterium tumefaciens*	Define the region integrated into the plant nuclear genome
Bacterial origin of replication; bacterial selection marker (backbone)	*E. coli* / *Pseudomonas aeruginosa* or equivalent	Maintenance and replication of the binary plasmid in bacteria prior to plant transformation; not integrated into the plant genome. Note: the reconstituted *TaExpA6* construct does not include hptI; no plant selectable marker is present in the T-DNA

CRISPR/Cas9 *GW2* / *GNI1-GNI2* editing construct – key elements

Element	Donor organism	Function
OsUbi promoter + intron	*Oryza sativa*	Drives constitutive expression of *Cas9* throughout the plant.
Cas9 coding sequence + 13 introns	*Streptococcus pyogenes* / *Arabidopsis thaliana*	Endonuclease generating RNA-guided double-strand breaks at *GW2* target sites. 13 introns added to *Cas9* cassette increase *Cas9* activity
TaU6 promoters (×2) + sgRNA scaffolds	*T. aestivum* (promoter); *S. pyogenes* (scaffold)	Guide RNAs directing *Cas9* to conserved sites in *GW2-A* (*TraesCS6A02G189300*), *GW2-B* (*TraesCS6B02G215300*), and *GW2-D* (*TraesCS6D02G176900*) homoeologs Also, *GNI-A2* (*TraesCS2A02G188500)*, *GNI-B2 (TraesCS2B02G218800)*, *GNI-D2 (TraesCS2D02G199200), GNI-A1 (TraesCSU02G009800)*, and *GNI-D1 (TraesCS2D02G385500)* *GNI-B1* (*TraesCS2B02G405700*) is confirmed absent in Paragon and Skyfall (and likely from Bamford)

Element	Donor organism	Function
hptI + *PvUbi2* promoter	*E. coli* / *Panicum virgatum L.*	Plant selectable marker (hygromycin resistance); also used as a molecular marker for detection of the CRISPR cassette by PCR
GRF4-GIF1 fusion cassette	*Triticum aestivum (TaGRF4)* / *Oryza sativa (OsGIF1)*	Morphogenic regulator cassette included to enhance Agrobacterium-mediated transformation efficiency and regeneration of transformed plants. *GRF4* encodes a GROWTH-REGULATING FACTOR transcription factor; *GIF1* encodes its GRF-INTERACTING FACTOR co-activator. This cassette promotes shoot organogenesis during tissue culture and is not associated with any intended agronomic trait in the regenerated plants.
NosT terminator (×2)	*Agrobacterium tumefaciens*	Transcriptional terminators for *Cas9* and *hptI*
T-DNA left and right borders	*Agrobacterium tumefaciens*	Define the region integrated into the plant genome

Part 4: 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

This application covers two complementary approaches to increase grain weight and yield in wheat, both centred on the expansin gene *TaExpA6* and the grain weight regulator *GW2*, as well as a third approach based on the floret fertility regulators *GNI1* and *GNI2*.

TaExpA6 – ectopic grain expression trait: Expansins are cell-wall loosening proteins that regulate cell expansion through turgor-driven stress relaxation in the cell wall (McQueen-Mason *et al.*, 1992). *TaExpA6* is an α-expansin that is normally expressed in wheat roots (Calderini *et al.*, 2021). In the pEW279-EXP construct and its reconstituted equivalent, *TaExpA6* is placed under the control of the wheat *puroindoline-b* (*PinB*) gene promoter, which drives transcription specifically in the endosperm, aleurone, and pericarp of developing seeds, but not in the embryo or vegetative tissues (Gautier *et al.*, 1994; Digeon *et al.*, 1999; Calderini *et al.*, 2021). *TaExpA6* is delivered to grain tissues in which it is not

normally active, targeting cell-wall loosening to early developing grain during pericarp and endosperm expansion. Critically, expression of the *PinB*::*TaExpA6* transgene in developing grains is absent at 5 days after anthesis (DAA) and peaks between 10 and 20 DAA after the critical period for grain number determination. This timing is key to avoiding the trade-off between grain weight and grain number (Calderini *et al.*, 2021; Vicentin and Calderini, 2024). RT-qPCR confirmed no transgene expression in roots or vegetative tissues of the original transgenic lines, and recombinant TaExpA6 protein abundance in developing grains was confirmed by LC-MS/MS proteomics (Calderini *et al.*, 2021). Ectopic overexpression of *TaExpA6* in grain under the *PinB* promoter increased average grain weight by up to 12.3% and grain yield by 11.3% in field experiments, without any negative effect on grain number (Calderini *et al.*, 2021; Vicentin and Calderini, 2024; Vicentin and Calderini, 2026). This trait was previously evaluated in glasshouse and field conditions by the Universidad Austral de Chile under SAG authorisation (event pEW279-EXP; SAG Resolutions 2833/2018 and 2899/2018), as part of the CRWT-funded project 'Wheat transformation to increase grain yield and industrial quality by improving kernel weight'. SAG is Chile's Agricultural and Livestock Service (Servicio Agrícola y Ganadero, SAG) and is the Chilean authority responsible for plant and animal health and the regulation of agricultural biotechnology.

GW2 – grain weight trait: The *GRAIN WIDTH2* (*GW2*) gene encodes a RING-type E3 ubiquitin ligase that acts as a negative regulator of grain size. Loss-of-function *gw2* mutations increase cell number and size in the grain pericarp, leading to larger, heavier grains (Simmonds *et al.*, 2016). EMS-induced or CRISPR-edited mutations in *GW2-A*, *GW2-B*, and *GW2-D* homoeologs act additively; the single A-genome mutant increases thousand grain weight (TGW) by approximately 6.6% and the triple mutant (*gw2-ABD*) by approximately 20% (Simmonds *et al.*, 2016; Wang *et al.*, 2018; Simmonds *et al.*, 2026). However, unlike *PinB*::*TaExpA6* overexpression, *gw2* loss-of-function acts before anthesis by increasing ovary size (Simmonds *et al.*, 2016; Vicentin and Calderini, 2024), overlapping with the critical period for grain number determination and resulting in a consistent trade-off: the triple mutant reduces both grains per spike and spike number per unit area, fully offsetting yield gains in multi-year, multi-location UK field trials (Simmonds *et al.*, 2026).

Combined *PinB*::*TaExpA6* × *gw2*; Rationale for combining both strategies: The combination of *PinB*::*TaExpA6* overexpression and *gw2* loss-of-function represents a complementary approach to increasing grain weight. *PinB*::*TaExpA6* acts post-anthesis in grain tissues, avoiding the grain number determination period and the associated trade-off. *GW2* loss-of-function acts pre-anthesis by increasing ovary size and grain capacity. Together, they may have additive effects on grain weight acting through different developmental windows and mechanisms. Field trials are required to evaluate whether combining these modifications delivers additive yield benefits in UK-adapted genetic backgrounds (Paragon, Skyfall, Bamford) and to characterise any pleiotropic effects under UK field conditions.

GNI1 and GNI2 – grain number trait: The *GRAIN NUMBER INCREASE1* and *2* (*GNI1* and *GNI2*) genes encode proteins belonging to the class I homeodomain leucine zipper family

(Sakuma et al., 2026). The GNI1 and 2 proteins exert an inhibitive growth function on the most apical floral primordia. *GNI2* is the ancestral gene, with *GNI1* being a more recent paralogous copy. Within UK germplasm, the *GNI-B1* gene appears to be deleted, leaving a total of five gene copies. Reduced-function mutations of individual homoeologs (e.g. *gni-A1*) have shown increased floral fertility and thus grain number, with grain yield increased by 10-30% (Sakuma et al., 2019). Characterising the effect of individual and multiple loss-of-function mutations at both *GNI1* and *GNI2* loci on grain yield in UK-adapted backgrounds (Paragon, Skyfall, and Bamford) requires field trials.

Lines for field release

Table of lines for field release. Lines will be released as they become available over the course of the release period; details of the exact edits to be released can be made ahead of the corresponding field season in the Notification of release Letter.

No.	Line Name	Type	Allele Combination
1	Paragon	Background Cultivar	WT
2	Skyfall	Background Cultivar	WT
3	Bamford	Background Cultivar	WT
4	Paragon_BC_WT	EMS gw2-ABD -*TaExpA6* BC_4	WT
5	Paragon_BC_*TaExpA6*	EMS gw2-ABD -*TaExpA6* BC_4	*TaExpA6*
6	Paragon_BC_gw2-A	EMS gw2-ABD -*TaExpA6* BC_4	gw2-A
7	Paragon_BC_gw2-A_*TaExpA6*	EMS gw2-ABD -*TaExpA6* BC_4	gw2-A+*TaExpA6*
8	Paragon_BC_gw2-AD	EMS gw2-ABD -*TaExpA6* BC_4	gw2-AD
9	Paragon_BC_gw2-AD_*TaExpA6*	EMS gw2-ABD -*TaExpA6* BC_4	gw2-AD+*TaExpA6*
10	Paragon_BC_gw2-ABD	EMS gw2-ABD -*TaExpA6* BC_4	gw2-ABD
11	Paragon_BC_gw2-ABD_*TaExpA6*	EMS gw2-ABD -*TaExpA6* BC_4	gw2-ABD+*TaExpA6*
12	Paragon_NULL	CRISPR gw2-ABD -*TaExpA6*	WT
13	Paragon_ gw2-ABD	CRISPR gw2-ABD -*TaExpA6*	gw2-ABD
14	Paragon_ gw2-ABD_alt	CRISPR gw2-ABD -*TaExpA6*	gw2-ABD
15	Paragon_ *TaExpA6*	CRISPR gw2-ABD -*TaExpA6*	*TaExpA6*
16	Paragon_ gw2-ABD_ *TaExpA6*	CRISPR gw2-ABD -*TaExpA6*	gw2-ABD+*TaExpA6*
17	Skyfall_NULL	CRISPR gw2-ABD -*TaExpA6*	WT
18	Skyfall_ gw2-ABD	CRISPR gw2-ABD -*TaExpA6*	gw2-ABD
19	Skyfall_ *TaExpA6*	CRISPR gw2-ABD -*TaExpA6*	*TaExpA6*
20	Skyfall_ gw2-ABD_ *TaExpA6*	CRISPR gw2-ABD -*TaExpA6*	gw2-ABD+*TaExpA6*
21	Bamford_NULL	CRISPR gw2-ABD -*TaExpA6*	WT
22	Bamford_ gw2-ABD	CRISPR gw2-ABD -*TaExpA6*	gw2-ABD
23	Bamford_ *TaExpA6*	CRISPR gw2-ABD -*TaExpA6*	*TaExpA6*
24	Bamford_ gw2-ABD_ *TaExpA6*	CRISPR gw2-ABD -*TaExpA6*	gw2-ABD+*TaExpA6*
25	Paragon_NULL	CRISPR gni1-gni2	WT
26	Paragon_ gni1	CRISPR gni1-gni2	gni1
27	Paragon_ gni2	CRISPR gni1-gni2	gni2
28	Paragon_ gni1_gni2	CRISPR gni1-gni2	gni1_gni2
29	Skyfall_NULL	CRISPR gni1-gni2	WT
30	Skyfall_ gni1	CRISPR gni1-gni2	gni1
31	Skyfall_ gni2	CRISPR gni1-gni2	gni2

No.	Line Name	Type	Allele Combination
32	Skyfall_ gni1_gni2	CRISPR gni1-gni2	gni1_gni2
33	Bamford_NULL	CRISPR gni1-gni2	WT
34	Bamford_ gni1	CRISPR gni1-gni2	gni1
35	Bamford_ gni2	CRISPR gni1-gni2	gni2
36	Bamford_ gni1_gni2	CRISPR gni1-gni2	gni1_gni2

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

TaExpA6 overexpression construct: The T-DNA insert spans 5,411 bp from the left to right border and contains the *PinB* promoter-*TaExpA6*-terminator transcriptional unit. It does not contain the *hptI* selectable marker.

CRISPR/Cas9 *GW2* editing construct: The T-DNA insert is 19,210 bp in size and contains all elements described in Section 12 above, including the *hptI* plant selectable marker. The presence of the transgenic cassette has been confirmed by PCR amplification of the *hptI* marker gene. Specific edits in *GW2-A*, *GW2-B*, and *GW2-D* have been confirmed by Nanopore next generation sequencing.

The presence of the *TaExpA6* transgenic insert in all *TaExpA6*-containing lines (BC4 and newly transformed) has been confirmed by PCR using primers spanning the *PinB* promoter-*TaExpA6* junction (Ta-PinB-F / TaExpA6-R; see Section 24).

CRISPR/Cas9 *GNI1+2* editing construct: The T-DNA insert is 19,210 bp in size and contains all elements described in Section 12 above, including the *hptI* plant selectable marker. The presence of the transgenic cassette has been confirmed by PCR amplification of the *hptI* marker gene. Specific edits in *GNI-A1*, *GNI-D1*, *GNI-A2*, *GNI-B2*, and *GNI-D2* have been confirmed by Nanopore next generation sequencing.

b. the size and function of the deleted region or regions

In CRISPR-edited lines, small insertions or deletions (InDels) are introduced at the Cas9 cut sites within the first exon of each *GW2* homoeolog (A, B, and D subgenomes). These InDels are predicted to cause frameshift mutations leading to premature stop codons and loss-of-function of the *GW2* E3 ubiquitin ligase. The sizes of the specific InDels per line and per homoeolog have been determined by NGS.

For *GNI1* and *GNI2*, the second exon was targeted to avoid highly conserved sequences with other class 1 homodomain leucine zippers in the first exon. The resulting InDels are predicted to cause frameshift mutations leading to premature stop codons and loss-of-function of the GNI1+2 class 1 homodomain leucine zippers.

Details of the exact edits to be released can be made ahead of the corresponding field season in the Notification of release Letter as some are still in development.

Cultivar	*GW2-A1*	*GW2-B1*	*GW2-D1*	*TaExpA6*
Paragon	91-bp deletion	92-bp deletion	91-bp deletion	No
Paragon	91-bp deletion	92-bp deletion	91-bp deletion	Yes
Paragon alt	1-bp del / 1-bp T insertion	92-bp deletion	2-bp del / 2-bp del	No
Skyfall	92-bp deletion	91-bp deletion	1-bp deletion	No
Skyfall	92-bp deletion	91-bp deletion	1-bp deletion	Yes
Bamford	in development	in development	in development	in development

All GW2 edits and EMS-induced mutations are homozygous within each subgenome. For the Paragon_alt line, the two entries shown per subgenome (e.g. GW2-A1: “1-bp del / 1-bp T insertion”) reflect independent edits at the two sgRNA target sites within that subgenome, both of which are homozygous.

Cultivar	*GNI-A1*	*GNI-D1*	*GNI-A2*	*GNI-B2*	*GNI-D2*
Paragon	1-bp G insertion	1-bp T insertion	138-bp deletion	1-bp deletion	138-bp deletion
Skyfall	in development	in development	in development	in development	in development
Bamford	in development	in development	in development	in development	in development

c. the copy number of the insert

Copy number analysis of the CRISPR transgenic cassette has been performed for the gw2, gni1/gni2 edited lines using quantitative PCR for the hptI marker gene. For the *PinB::TaExpA6* lines, the construct does not contain the hptI gene and molecular copy number determination has not been performed. Homozygosity of the *TaExpA6* insert in lines selected for field release has been inferred from segregation analysis, with the absence of null segregants among progeny indicating stable homozygous inheritance. The transgenic elements present in the insert are not expected to represent a risk to the environment regardless of copy number (see Part IVa).

d. the location or locations of the insert or inserts in the plant cells

Both the *TaExpA6* and CRISPR constructs were introduced using Agrobacterium-mediated transformation and are therefore integrated in the nuclear genome. CRISPR-induced edits are at the endogenous *GW2*, *GNI1*, and *GNI2* loci 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

Segregation analysis by PCR across multiple generations (T2 onwards) using the *PinB*::*TaExpA6* junction primers (Ta-PinB-F / TaExpA6-R) confirms that the *TaExpA6* transgene insert is stably inherited in the nuclear genome of all selected lines. Lines selected for field release are homozygous. The CRISPR-induced *GW2*, *GNI1*, and *GNI2* edits have been confirmed as stable and heritable, with no evidence of further editing in subsequent generations. Phenotypic observations in glasshouse-grown plants confirm consistent increases in grain size in *TaExpA6*-overexpressing lines and *gw2* EMS loss-of-

function lines, as expected from the intended modifications. Phenotyping will be conducted as fixed homozygous plants become available.

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

The *TaExpA6* gene is under the transcriptional control of the endosperm-specific *PinB* promoter. In the original transgenic lines (event pEW279-EXP, cv. Fielder), expression of the transgene was verified by RT-qPCR using transgene-specific primers, with elevated transcript levels in developing grain tissue at 10–20 days after anthesis and no detectable expression in roots or vegetative tissues (Calderini et al., 2021). Recombinant TaExpA6 protein abundance in developing grains was confirmed by LC-MS/MS proteomics (Calderini et al., 2021).

The *GW2*, *GNI1*, and *GNI2* CRISPR edits have been confirmed by Nanopore next generation sequencing. The molecular characterisation data confirm that the intended modifications are present, stable, and performing as expected.

Part 4a: 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 *TaExpA6* overexpression and *GW2*, *GNI1*, and *GNI2* loss-of-function modifications are intended to increase grain weight and grain number, respectively. Neither modification is expected to confer any selective advantage in unmanaged ecosystems, increase plant competitiveness relative to wild plants, or alter the persistence, invasiveness, or ability to establish in uncultivated environments. Wheat is not naturalised in the UK and does not establish persistent populations outside of cultivation. Critically, the *PinB* promoter driving *TaExpA6* expression is specifically active only in the developing grain endosperm and is silent in all vegetative tissues, meaning that any growth or competitive advantage conferred by *TaExpA6* is restricted to grain fill and would not affect vegetative competitiveness.

Glasshouse observations indicate that the GM and gene-edited lines have normal vegetative phenotypes, with no obvious changes in plant height, flowering time, or overall plant vigour relative to untransformed controls. Plants remain sensitive to all conventional herbicides. Management procedures including a 2 m pollen barrier surrounding the trial plots and a minimum 20 m separation to other non-GM cereals will further minimise the probability of cross-pollination.

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

The risk of horizontal gene transfer from the integrated T-DNA to soil microorganisms is considered extremely low and consistent with that described for other Agrobacterium-

mediated wheat transformants previously released in UK field trials (e.g. consent 19/R52/02; 21/R52/01). In *TaExpA6* lines, the *hptII* gene is absent from the T-DNA, removing concerns about transfer of antibiotic resistance genes from these lines. In CRISPR *gw2*, *gni1*, and *gni2* lines, the *hptII* gene is present and integrated in the plant nuclear genome; in the unlikely event of horizontal gene transfer, expression in soil microorganisms would be improbable as the gene is under the control of plant-specific promoters. The *hptII* gene is classified in the EFSA Group 1 antibiotic resistance marker genes for which no restrictions are required in field experimentation or marketing.

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

No changes in interactions with non-target organisms are predicted. The *PinB* promoter restricts *TaExpA6* overexpression to the grain endosperm; the protein is therefore not present in vegetative or root tissues (beyond in its wild-type, native level) where interactions with soil organisms, insects, or pathogens would occur. The *GW2* E3 ubiquitin ligase affects grain development internally. Likewise, the *GNI1* and *GNI2* class I homeodomain zipper transcription factors control flower fertility within the wheat spikelet. Neither modification is expected to produce novel compounds that would interact with non-target organisms in the field environment.

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

No changes to agricultural practice or management are predicted as a result of the genetic modifications. The field trial will be conducted using standard agronomic practices appropriate for wheat cultivation at the release site.

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

No interactions with the abiotic environment beyond those of conventional wheat are predicted. The genetic modifications are targeted at grain development processes and are not expected to alter responses to soil conditions, temperature, water availability or other abiotic factors.

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

TaExpA6 is a native wheat protein belonging to the expansin family, which are widely present across the plant kingdom. There are no known or predicted toxic or allergenic properties associated with this protein. Its overexpression is restricted to the grain endosperm by the *PinB* promoter. Similarly, *GW2* is a native wheat protein; loss-of-function mutations in *GW2* would reduce or eliminate this protein in grain tissue but are not expected to generate novel toxic or allergenic compounds. *GNI1* and *GNI2*, likewise, are native wheat proteins; loss-of-function mutations in *GNI1* and *GNI2* would reduce or eliminate these proteins from seedling, spike, and grain tissues but are not expected to generate novel toxic or allergenic compounds.

In CRISPR *gw2*, *gni1*, and *gni2* lines, the *hptII* gene product (hygromycin phosphotransferase) is present and classified by EFSA as a Group 1 antibiotic resistance marker gene; EFSA has indicated that no restrictions are required for field experimentation. In *TaExpA6*-only lines, the *hptII* gene is absent from the T-DNA, so there is no hygromycin resistance marker in those plants.

The plant lines and any grain harvested from them will not enter the food or feed chains. Potential toxic or harmful effects on human health arising from the genetic modification are considered negligible.

23. Conclusions on the specific areas of risk

In summary, neither the *PinB*::*TaExpA6* overexpression nor the *GW2*/*GNI1*/*GNI2* loss-of-function modifications are expected to result in any adverse effects on human health or the environment. The *PinB* promoter ensures that *TaExpA6* overexpression is strictly limited to the grain endosperm. The proteins involved are of plant origin, the modifications are targeted at grain and floret development, and the trial will be conducted at small scale under strict biosecurity conditions. The risk of horizontal gene transfer is assessed as very low. Based on the analyses provided above, the overall risk of harm to human health or the environment arising from this trial is assessed as very low.

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

TaExpA6 lines

Two independent PCR approaches are available for detection of *TaExpA6* transgenic lines, as described below. As *TaExpA6* lines do not contain *hptII*, these PCR-based approaches are the primary detection methods for these lines.

(i) PinB promoter–TaExpA6 junction PCR (primary method for transgene presence/absence)

A primer pair spanning the junction between the *PinB* promoter of the transgene and the *TaExpA6* coding sequence gives a diagnostic band specific to the transgenic construct, which is not amplified from the endogenous *PinB* locus. Primers:

- Forward: Ta-PinB-F 5′-ACAACACACAATGGTAGGCAAA-3′
- Reverse: TaExpA6-R 5′-GGTCCCCTTCACCGACAT-3′

(ii) TaExpA6 A-genome-specific (ExpA6_A_spec) intron-spanning PCR (confirmatory method)

A-genome-specific primers targeting *TaExpA6* were designed to span an intron present in the genomic sequence. This allows discrimination between the transgenic (cDNA-based, intronless) and wild-type (genomic, intron-containing) alleles by amplicon size on an agarose gel. In transgenic plants the PCR product is smaller (no intron) than in wild-type plants.

Expected amplicon sizes:

- Wild-type (genomic, with intron): 843 bp
- Transgenic (cDNA-derived, intronless): 710 bp

Primers:

- Forward: ExpA6_A_spec_F 5′-CTTCGTGCAGGCCAGG-3′
- Reverse: ExpA6_A_spec_R 5′-GTACCGTTGTCAAGCTGATGA-3′

CRISPR *gw2* lines

The presence of the CRISPR transgenic cassette is detected by PCR amplification of the *hptII* hygromycin resistance marker gene.

CRISPR-induced *GW2* edits are detected by PCR amplification encompassing the CRISPR target sites, followed by next-generation sequencing (NGS) to confirm and quantify the specific InDel(s) at each homoeolog (*GW2-A*, *GW2-B*, and *GW2-D*). Primers for GW2 edit detection (NGS):

- Forward: GW2_NGS_GE_Fw1 5′-TGTATCTACAGTGAGGCGGC-3′
- Reverse: GW2_NGS_GE_Rev2 5′-CCCAATGAGATGCAAGCCAC-3′

These primers amplify a region spanning the CRISPR cut sites in the first exon of *GW2* homoeologs. NGS of the amplicon allows identification and quantification of InDels at all three subgenomes (A, B, and D) in a single reaction and confirms homozygosity of each edit in lines selected for field release.

CRISPR *gni1* and *gni2* lines

The presence of the CRISPR transgenic cassette is detected by PCR amplification of the *hptII* hygromycin resistance marker gene.

CRISPR-induced *GNI1* and *GNI2* edits are detected by PCR amplification encompassing the CRISPR target sites, followed by next-generation sequencing (NGS) to confirm and quantify the specific InDel(s) at each homoeolog (*GNI-A1*, *GNI-D1*, *GNI-A2*, *GNI-B2*, and *GNI-D2*). Primers for *GNI1+2* edit detection (NGS):

- Forward: GNI1_2_ONT_F1 5′- TTTGCGCATGCGATGATG-3′
- Reverse: GNI1_2_ONT_R1 5′- TGAAACCCGGATGCTGCT-3′

These primers amplify a region spanning the CRISPR cut sites in the second exon of all *GNI1* and *GNI2* homoeologs. NGS of the amplicon allows identification and quantification of InDels at all three subgenomes (A, B, and D) and each of the genes (*GN1* and *GN2*) in a single reaction and confirms homozygosity of each edit in lines selected for field release.

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

The *TaExpA6* overexpression event (pEW279-EXP) has been previously authorised for field evaluation in Chile by the Servicio Agrícola y Ganadero (SAG) under Resolution Exenta Nº 2899/2018 (authorising retention in-country and field sowing) and Resolution Exenta Nº 2833/2018 (authorising environmental release and establishing biosecurity measures). These trials were conducted by the Universidad Austral de Chile at Valdivia as part of the CRWT-funded project 'Wheat transformation to increase grain yield and industrial quality by improving kernel weight'. No adverse effects on human health or the environment were identified during those trials.

No previous releases of the CRISPR *gw2*/*gni1*/*gni2*-edited lines or of the combined *PinB*::*TaExpA6* × *gw2* lines have been made in the UK or elsewhere.

Part 5: Information relating to the site of release

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

The transgenic plants will be released on an area of arable land at Church Farm, Bawburgh which is owned and operated by JIC (Ordnance Survey map grid reference TG 149 087). It will be situated within the fenced area (110m x 160m) of Football Field which is dedicated for use of GM experiments (total area of 17,600 m2).

The trial area will comprise a maximum of 168 (6m^2) plots coming from *PinB*::*TaExpA6*, CRISPR *gw2*, or a combination of both, CRISPR *gni1*/*gni2* and non-GM lines: cvs Paragon, Bamford, Skyfall, and Soissons as controls. As material becomes available over time smaller 1m^2 plots will make up a proportion of the trial area to bulk up seed for the yield scale plots. In accordance with wheat planting practice, the trial site will rotate within the release site each year of the trial, with a maximum experimental area of 1,543 m² per growing season. In Year 1 (2027), both a Spring sowing (March/April 2027) and an Autumn sowing (October 2027) will take place within the same calendar year on separate areas of the site; the combined trial area in Year 1 will therefore not exceed 3,086 m².

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 3,748 m² in year 1. In Years 2–5, a single sowing per calendar year will be made, with a trial area not exceeding 1,543 m² and a total area including pollen barrier of 1,874 m² per year.

In each year, no cereals (other than those cultivated either as part of this consent or under a separate consent to release a GMO) or grass species will be cultivated or allowed to grow within 20 m from the outer edge of the release area.

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

The release site (Ordnance Survey map grid reference TG 149 087) is arable land located at the John Innes Centre (JIC) Church Farm in Bawburgh, Norfolk. Some areas are bordered by deciduous hedges or trees. Flora in the immediate vicinity will be unknown until decisions on other local (non-GM) field trials are made each year but will likely be limited to cereals (wheat/barley) and oilseed rape. Except for the experimental plots themselves, no cereal crop (other than those cultivated either as part of this consent or under a separate consent to release a GMO) will be grown within the accepted separation distance of 20 metres from the release site. The fauna is typical of agricultural land in the 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 *Agropyron*) although there are no reports of cross-hybridisation between wheat and these genera. The two most common inland species are *Elytrigia repens* (common couch grass = *Agropyron repens*) and *Elymus caninus* (bearded couch = *Agropyron caninum*). Other related species, such as *Elytrigia juncea* (Sand couch = *Agropyron junceum*), *Elytrigia atherica* (Sea couch = *Agropyron pycnanthum*) and hybrids are largely confined to coastal habitats.

E. repens is not common but can be found around the Church Farm site whereas *E. caninus* is uncommon in Norfolk (National Biodiversity Network). *E. repens* propagates primarily by vegetative reproduction (rhizomes), rather than by sexual reproduction, and in any case, no reports of wheat x *Elytrigia* or *Elymus* spontaneous hybrids have been reported. *E. repens* will be controlled along with other weeds in and around the trial site using standard farm practices. No wheat or other cereals, including *E. repens* will be cultivated or allowed to grow within 20 meters from the trial. Presence of any cereal or *E. repens* in the 20 m surrounding area will be removed either by hand pulling or application of a glyphosate herbicide between 1st May and 30th September in each year of the trial.

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

There are no officially recognised biotopes, protected areas or Sites of Special Scientific Interest (SSSIs) within 3.5 km of the release site. The closest SSSI to the release site are the River Wensum (~3.5 km away) and Sweet Briar Road Meadows (~5.5 km away). The fact that wheat is not a wind-pollinated crop and is not visited frequently by pollinators means that there is minimal risk to any officially recognised biotopes or protected areas listed by Natural England.

Part 6: 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 purpose of these field trials is to evaluate the agronomic performance, grain weight, grain quality, grain number, and yield of wheat lines carrying the *PinB*::*TaExpA6* overexpression transgene and/or *GW2*/*GNI1*/*GNI2* loss-of-function mutations under UK field conditions. The trials will specifically investigate:

- The effect of endosperm-specific *TaExpA6* overexpression (driven by the *PinB* promoter) on grain weight, grain dimensions and yield components in UK-adapted wheat cultivars (Paragon, Skyfall, Bamford).
- The additive or synergistic interaction between *PinB*::*TaExpA6* overexpression and *GW2* loss-of-function on grain weight and yield, across single, double and triple homoeolog mutant combinations.
- The agronomic stability and performance of BC4 Paragon backcross lines carrying combinations of *PinB*::*TaExpA6* and *gw2* mutations.
- The effect of multiple *GNI1* and *GNI2* loss-of-function mutations on grain number within UK-adapted wheat germplasm.

Grain harvested from the trials will be used for laboratory analysis of grain composition and quality parameters. There is no current intention to use these lines as commercial products; the trial is a research activity to generate proof-of-concept data to inform future plant breeding strategies aimed at improving wheat yield.

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

If consent is granted, the first field trial will start in Spring 2027. Subsequent sowings will be made in either autumn (October) or spring (March) depending on the varietal background and growth habit of each line. All plants will be harvested in July/August, with the exact timing dependent on weather conditions and crop maturity at the time.

The trial is designed to accommodate lines becoming available at different stages as seed multiplication and genotyping are completed. In Spring 2027, the EMS-derived *gw2-ABD* × *PinB*::*TaExpA6* BC4 lines (lines 1 and 4–11) will be ready for sowing, 17 genotype entries in total, each sown in single or replicated plots depending on seed availability at the time.

Limited seed quantities of the CRISPR-derived *gw2-ABD* × *PinB*::*TaExpA6* lines (lines 2, 12–16 and 17–20) should also be available for initial small-plot (1 m²) assessment at this stage.

By Autumn 2027 and Spring 2028 (year 2), all remaining lines will be entering the trial for the first time, with plots sizes at either 1 m² for initial assessment or 6 m² for yield evaluation as seed availability permits. This staged entry means that years 1 and 2 serve primarily as initial assessment and seed bulking seasons, with the full complement of lines sown at yield-plot scale from year 3 onwards. Growing seasons 3–5 (2029–2031) will therefore provide three full consecutive years of replicated yield-scale assessment across all lines, which is the minimum required to draw robust conclusions about yield performance across seasons and environments. We therefore propose five growing seasons in total, with five harvests from 2027 to 2031.

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

The 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 trial site will be prepared by the Field Experimentation staff, 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 July or August, once all the plants have reached maturity. 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 threshed and stored appropriately in GM seed containers. The plot combine will be cleaned on the field at the end of harvesting to ensure no grain is removed from the trial site. Remaining plant material will be chopped and left *in situ* with the stubble, which will be cultivated post-harvest. Any unrequired seed from the experimental plots or pollen barrier will be placed in sealed, labelled bags or containers and disposed of in accordance with JIC standard operating procedures for transgenic material. Transportation of waste materials will be in secure containers. All procedures will be undertaken to strict SOPs developed within release 21/R52/01.

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

Seeds will be drilled at a sowing density of 300 plants m^{-2}. The trial will comprise a maximum of 44 experimental lines per release, including two copies of lines 4-11 to account for the *PinB*::*TaExpA6* ± *gw2* combinations, of which three are non-GM UK

cultivar controls (Paragon, Skyfall, Bamford). The remaining 41 GM lines will each be sown in three replicate plots of 6.24 m², giving a maximum of 123 GM plots per release. At a sowing density of 300 plants m^{-2}, this gives a maximum of 230,256 GM plants per release.

In Year 1 (2027), both a Spring sowing (March/April 2027) and an Autumn sowing (October 2027) will take place within the same calendar year on separate areas of the site (see Section 26). As each constitutes a separate release, the maximum number of GM plants to be released in Year 1 is therefore 460,512, comprising two releases of up to 230,256 GM plants each. In Years 2 to 5, a single release per calendar year will be made, with a maximum of 230,256 GM plants per year.

Part 7: Information on control, monitoring, post-release and waste treatment plans

35. A description of any precautions to maintain spatial and temporal separation of the genetically modified plant from sexually compatible plant species, especially a. wild and weedy relatives, b. crops

Wild and weedy relatives: 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 *Agropyron*) although there are no reports of cross-hybridisation between wheat and these genera. The two most common inland species are *Elytrigia repens* (common couch grass = *Agropyron repens*) and *Elymus caninus* (bearded couch = *Agropyron caninum*). Other related species, such as *Elytrigia juncea* (Sand couch = *Agropyron junceum*), *Elytrigia atherica* (Sea couch = *Agropyron pycnanthum*) and hybrids are largely confined to coastal habitats.

E. repens is not common but can be found around the Church Farm site whereas *E. caninus* is uncommon in Norfolk (National Biodiversity Network). *E. repens* propagates primarily by vegetative reproduction (rhizomes), rather than by sexual reproduction, and in any case, no reports of wheat x *Elytrigia* or *Elymus* spontaneous hybrids have been reported. *E. repens* will be controlled along with other weeds in and around the trial site using standard farm practices. No wheat or other cereals, including *E. repens* will be cultivated or allowed to grow within 20 meters from the trial. Presence of any cereal or *E. repens* in the 20 m surrounding area will be removed either by hand pulling or application of a glyphosate herbicide between 1st May and 30th September in each year of the trial.

Crops: 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, 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. Pollen will be allowed to be produced as we require grain but it's short period of viability and the separation distance of the transgenic plants from other wheat crops (at least 20 metres) will minimise the risk of cross-pollination.

Any equipment used during the growing season, including for planting and harvesting of transgenic material, will be thoroughly cleaned after use and before it is allowed to leave the release site.

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. All procedures will be undertaken to strict SOPs developed within release 21/R52/01.

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

The ears of all experimental plants will be collected using a plot combine and seeds will be threshed and stored in appropriate GM seed containers for use in yield-related trait analyses.

The pollen barrier will be harvested using a plot combine. The front and back of the pollen barrier will be harvested first to enable access to the experimental plots, with the sides harvested last, ensuring that non-GM pollen barrier material is always combined after the GM experimental plots.

During combining, seeds will be retained and remaining plant material will be chopped and left *in situ* with the stubble, which will be cultivated post-harvest. Any regrowth or volunteer plants will be destroyed with glyphosate.

Any unrequired seed from either the pollen barrier or experimental plots will be placed in sealed, labelled bags or containers and disposed of in accordance with JIC standard operating procedures for transgenic material.

38. A description of monitoring plans and techniques

The purpose of the monitoring plan is to enable early detection of any unintended effects related to the release of the transgenic wheat plants.

The release site will be visited by trained personnel who are working on the project once each week and records will be kept of each visit. Any unexpected occurrences that could potentially result in adverse environmental effects or the possibility of adverse effects on human health will be notified to the national inspectorate immediately. Should the need arise to terminate the release at any point the emergency plans detailed below will be followed.

Post-trial the release site will remain fallow to enable easy identification of volunteers. The site (plot and the 20m border) will be inspected once every week between harvest and the end of November of the relevant year and then once a month from 1 March until 31 August in the following two years. We will record the number of volunteers detected in each month before they are controlled in either by application of a systemic herbicide or by hand pulling plants and digging out the root systems. These will then be autoclaved within JIC. If volunteers are found at the end of the 2-year period, DEFRA recommendations will be followed for the management of the release site. We will refrain from cultivating cereal crops intended to enter the food and/or feed chain on the trial site until monitoring of the plots for volunteers has ended.

39. A description of any emergency plans

In the unlikely event that the integrity of the site is seriously compromised, we will take immediate and appropriate preventative and remedial action. If required, the trial will be terminated, and plants will be destroyed using a suitable herbicide or harvesting as deemed appropriate. All harvested material will be removed from the site, placed in sealed, labelled bags or containers, and disposed of by incineration using our approved contractor. Transportation of waste materials will be in secure containers. We will proceed to notify the Secretary of State of the emergency as soon as practicable and in any event within thirty-six hours of the matter constituting the emergency, detailing the nature of the emergency and any action that has been taken. We will also submit a plan to the Secretary of State for his approval as soon as practicable and in any event within forty-eight hours of the matter constituting the emergency, detailing any continued or further action that he proposes to take to restrict the dispersal of the GMO from the trial site. The phone numbers of all key staff will be available to site security and field personnel.

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. Audible and visual deterrents will also be deployed to minimise access by birds and other animals during the growing season.

The JIC trials teams have experience of previous GM field trials and the relevant management procedures at those sites. GM inspectorate will have access to the trial sites on request.

Part 8: 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 *PinB*::*TaExpA6* overexpression construct (pEW279-EXP) was generated as described in Caderini *et al.* (2021).

The construct was also generated at the John Innes Centre and introduced into wheat by Agrobacterium-mediated transformation of immature embryos, following the protocols described in Hayta *et al.* (2019; 2021). Transformation was performed by the John Innes transformation facility.

2. Detection and genotyping of *TaExpA6* lines was performed by PCR using primers Ta-PinB-F (5′-ACAACACACAATGGTAGGCAAA-3′) and TaExpA6-R (5′-GGTCCCCTTCACCGACAT-3′), which amplify a diagnostic fragment spanning the *PinB* promoter–*TaExpA6* junction of the transgene.

3. The CRISPR/Cas9 *GW2* editing construct was assembled by Golden Gate MoClo assembly as described in Smedley *et al.* (2021). sgRNA sequences were either from Wang *et al.* (2018) or designed using the WheatCRISPR tool. Transformation of immature embryos was performed by the John Innes transformation facility following the same Agrobacterium-mediated protocols as described above.

4. The CRISPR/Cas9 *GNI1*/*GNI2* editing construct was assembled by Golden Gate MoClo assembly as described in Smedley *et al.* (2021). sgRNA sequences were designed using the WheatCRISPR tool. Transformation of immature embryos was performed by the John Innes transformation facility following the same Agrobacterium-mediated protocols as described above.

5. Detection of the CRISPR transgenic cassette was by qPCR using primers specific to the *hptII* gene. CRISPR-induced edits in *GW2-A*, *GW2-B*, and *GW2-D*, as well as *GNI-A1*, *GNI-D1*, *GNI-A2*, *GNI-B2*, and *GNI-D2*, were detected by PCR amplification encompassing the CRISPR target sites, followed by next-generation sequencing (NGS) to confirm and quantify the specific InDel(s) at each homoeolog (*GW2-A*, *GW2-B*, *GW2-D*, *GNI-A1*, *GNI-D1*, *GNI-A2*, *GNI-B2*, and *GNI-D2*) performed at the John Innes Centre.

6. The BC_4 backcross programme was carried out the John Innes Centre. Genotyping across backcross generations was performed by *PinB*::*TaExpA6* junction PCR to confirm presence/absence of the *TaExpA6* transgene and to select the desired *gw2* mutation combinations.

7. Glasshouse growth, generation advancement and seed bulking were performed at the John Innes Centre. Phenotypic assessments (plant morphology, thousand grain weight, grain number) was carried out according to standard methods.

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