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 Crop Science Centre, University of Cambridge/National Institute of Agricultural Botany (NIAB) at the following address:

Crop Science Centre
Department of Plant Sciences
University of Cambridge
93 Laurence Weaver Road
Cambridge, CB3 0LE, UK

Persons responsible for planning and carrying out release and carrying out supervision, monitoring and safety of the release:

Research scientist, lead applicant	Russell R Geiger Professor of Crop Science, Director of Crop Science Centre, Scientist who has led research on plant- microbe interaction and plant biotechnology for over two decades, BA in Plant Biology (University of East Anglia), PhD (University of California, Berkeley) and Postdoctoral Fellow in symbiotic associations (Howard Hughes Medical Institute at Stanford University, California)
Research Scientist	Postdoctoral Research Associate and field trials co-ordinator, Crop Science Centre, MBiolSci (University of Sheffield), PhD (University of York), and 10 years' experience growing barley at University of Sheffield,

	University of York, University of Leeds and University of Cambridge.
Director of Research Management, NIAB	35 years of agricultural and agricultural field trials experience. Now managing 800ha of land in Cambridgeshire and Kent for the sole purpose of trials work on Arable and Horticultural crops.
Impact Manager	Over 10 years of experience in plant molecular biology and biotechnology, PhD (University of Western Ontario, Canada), Postdoctoral Research Associate (University of Cambridge)

2. The title of the project.

Field assessment of arbuscular mycorrhizal contributions using gene-edited and gene-modified spring barley

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 *Hordeum*

(c) species *Hordeum vulgare*

(d) subspecies

(e) cultivar/breeding line Golden Promise

(f) common name barley, malting barley

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 seeds. The fertile florets consist of both male and female reproductive structures, and fertilization occurs as the spikes are

emerging from the boot. Barley is predominantly self-pollinated and autogamous under natural field conditions, with self-fertilization normally occurring before flowers open. There is no self-incompatibility system, making barley a natural inbreeder with low rates of outcrossing (<5%) [1]. Barley seeds are approximately eight millimetres in length and weigh approximately fifty milligrams when mature. The 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

Final grain yield is largely determined by grain number, which is set by tiller survival and fertile spikelet number and survival. Pollination, seed-set, and grain filling are dependent on moisture stress, temperature, weather conditions, agronomic practices, herbicides, nutrition, and pressure applied by pests and disease.

(iii) generation time; and

As a spring-barley type, one season for Golden Promise is usually from late February to no later than September of 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.

Barley is naturally an inbreeding hermaphrodite plant, but it can be crossed with wild grasses under experimental conditions in the field and the glasshouse, such as wild barley, *Hordeum spontaneum*. This species is confined to the south-eastern area of Europe, North Africa and Asia. No reports of hybrids between *H. spontaneum* and *H. vulgare* are known in the UK. Barley can also be forced under laboratory conditions to cross with a small number of cultivated cereals including rye and wheat, but if such crosses occur spontaneously in the field, they must be extremely rare.

5. Information concerning the survivability of the plant:

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

Barley is an annual grass species and survives from year to year only through seed production. In common agronomic practice, mature seeds may fall from the plant

before or during harvest and not be collected. These seeds may germinate in the same autumn, growing as volunteers. A very small number of the discarded seeds may overwinter in a dormant state and germinate the following spring, growing as volunteers [2].

(b) any specific factors affecting survivability.

Golden Promise is a UK milling barley variety with low frost tolerance, which means it is sown as a spring-type under typical UK conditions.

6. Information concerning the dissemination of the plant:

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

Pollen can be disseminated by wind, but this is limited by the size and weight of the pollen grain. The risk of cross-pollination is also reduced by its short period of viability. There is a very low level of outcrossing in natural and cultivated barley populations <5% [1]. Outcrossing can increase if plants are exposed to stress just prior to and/or during the spike (ear) emergence phases [3]. Reports quantifying the rate of cross-pollination state that out-crossing rates are usually less than 1.8% among different barley populations [4]. Studies suggested that outcrossing may vary considerably among seasons and that high precipitation and cool temperatures during flowering tend to enhance outcrossing [4].

(b) any specific factors affecting dissemination.

Seed is usually retained by the plant until harvest, but a small proportion can be shed to the ground at that time. Dispersal of seed before harvest by wind is unlikely but possible by wildlife.

7. The geographical distribution of the plant in Europe.

Barley is grown in over 100 countries worldwide, mainly in temperate zones but also in some tropical regions. Barley is typically sown right across the European continent with Germany, France, Ukraine, Spain, and the United Kingdom being major barley producers. In 2019, the EU country members harvested over 55.6 million tonnes of barley (https://ec.europa.eu/eurostat/statistics-explained/index.php?title=Agricultural_production_-_crops)

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 – barley is grown in Europe

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.

Barley plants interact with a range of beneficial and pest insects and fungal pathogens. Barley can become infected by several fungal foliar pathogens in the UK including brown rust (*Puccinia hordei*), net blotch (*Pyrenophora teres*), powdery mildew (*Blumeria graminis sp. hordei*), ramularia (*Ramularia collocygni*), Rhynchosporium (*Rhynchosporium commune*) and yellow rust (*Puccinia striiformis*). Colonisation by arbuscular mycorrhizal fungi (AMF) has been demonstrated in some cases to enhance host plant defence against pathogens [5], although data is scarce for barley. Reduction in capacity for AMF to colonise barley roots (as in gene-edited mutant lines such as *symRK*) may result in slightly increased susceptibility to foliar fungal pathogens. Barley interacts with multiple protists, bacteria, and fungi in the rhizosphere [6, 7]. The interaction with AMF is expected to be altered in the genetically modified and genetically-edited plant lines.

Barley is not toxic and is the fourth most significant grain in the world and is considered an important source for food, feed, and malt. However, like wheat, it may cause gastro-intestinal intolerance, coeliac disease, and/or ‘bakers’ asthma’ in susceptible individuals [World Allergy Organization White Book on Allergy]. This is not expected to be affected in any way by the traits carried by the plants in this trial. Plants and seeds 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 study aims to undertake a field trial to examine the impacts of arbuscular mycorrhizal fungi (AMF) inoculation on biomass and yield of the symbiosis pathway gene-edited and the overexpression gene-modified spring barley cv. Golden Promise in low and rich phosphate soils. For this field trial, six of the barley cv. Golden

Promise genes involved in the perception of AMF, namely *SYMRK*, *CCamK*, *Cyclops*, *RAM1*, *NSP1*, and *NSP2* have been individually edited using the CRISPR-Cas9-mediated gene editing system. In this release, 11 independent lines in which one of the aforementioned genes was edited will be used. Genetic edition in these genes led to abortion or significant reduction of AMF colonization in the laboratory conditions. Moreover, one of the said genes, namely *Hordeum vulgare NSP2* (*HvNSP2*) and its ortholog from *Medicago truncatula* (*MtNSP2*), were modified through overexpression (Ox) which led to the promotion of AMF colonization in the laboratory setting.

The methods used to create barley CRISPR gene-edited lines are as described [8]. The production of the gene-edited plants required the introduction of *Cas9* which is an RNA-guided endonuclease associated with the CRISPR (Clustered Regularly Interspersed Short Palindromic Repeats) type II adaptive immune system. The *Cas9* endonuclease and a single guide RNA (sgRNA) cas9/sgRNA complex is able to interrogate the genome sequence and introduce double-strand breaks (DSB) at a particular locus which are then repaired by the host cells own error-prone non-homologous end joining mechanisms (NHEJ). For each of the six target genes released in this field trial, we made two dual sgRNAs, namely pair 1A, 1B, 2A, and 2B each with a pair of unique sgRNAs, such that, 23 nucleotide sequences targeting uniquely the exons of these six genes were identified. To select the target sequences, the barley genome sequence hosted by Deskgen.com was used. BLAST searches against the barley genome to check off-targets were done at Ensembl Plants http://plants.ensembl.org/index.html and at morexGenes hosted by The James Hutton Institute https://ics.hutton.ac.uk/morexGenes/blast_page.html. The specific target sequences of guide RNA for each gene-edited lines in this release are listed in Table 1. Note that in these release, the *cyclops-2* and *cyclops-3* gene-edited lines were resulted from the same pair of guide RNA (Pair1). Similarly, *nsp2-2* and *nsp2-4* gene-edited lines were developed from the same pair of guide RNA (Pair2).

To introduce CRISPR/Cas9 targeted gene editing, we used four transcriptional Goldengate Level 1 cassettes (Figure 1). Adjacent to the left border was the first cassette that provided resistance to hygromycin and allowed regeneration of barley plants which contain the T-DNA integrated into the genome. The second cassette expressed nuclear-localized Cas9 in a ubiquitous fashion while the third and fourth cassette utilized the wheat U6 promoter to drive transcription of sgRNA specific to the relevant target locus. These four Level 1 constructs were assembled to create one donor Level 2 donor vector containing dual sgRNAs following the standard protocol [9]. The plasmid clones were verified by restriction enzyme digestions and sequencing. These constructs were transformed into *Agrobacterium tumefaciens* AGL1 in preparation for barley transformation.

The T-DNA binary vectors were then transformed through the routine transformation protocol for the spring barley Golden Promise [10 & 11], based on *Agrobacterium*-

mediated inoculation of immature embryos as described [12]. Briefly, barley spikes containing immature seed were collected and a single seed from the middle of the spike was carefully removed. The seeds were sterilized and immature embryos were isolated and later co-cultivated with the *Agrobacterium* inoculation containing the desired plasmids. The callus was then induced with the selection of antibiotic hygromycin. Subsequently, the immature-embryos-derived-calli were chosen for regeneration and rooting. The T0 plants with long enough roots and shoots were moved to soil to be grown to maturity and tested for the number of transgenes. The presence of the introduced T-DNA was confirmed by PCR and transgene copy number determined by Quantitative Real-time PCR. T0 genetically modified plants that contain only one copy number of the inserted gene were chosen to be taken forward. Homozygous T1 plants were taken forward.

Table 1. The specific target sequences of guide RNA used to create the 11 gene-edited lines in this release. Note that two independent dual guide RNA (Pair1 and Pair 2) have been used for *ccamk*, *ram1*, and *nsp1* genes resulting into the development of *ccamk-1*, *ccamk-2*, *ram1-1*, *ram1-2*, *nsp1-1*, and *nsp1-4* gene-edited lines, respectively. Moreover, the *cyclops-2* and *cyclops-3* gene-edited lines were resulted from the same pair of guide RNA (Pair1) and *nsp2-2* and *nsp2-4* gene-edited lines were alsodeveloped from the same pair of guide RNA (Pair2).

Line description	gRNA Pair Number	Guide A sequence	Guide B sequence
symrk-2	Pair 1	ccccatctgcctgggaaggcttc	gggccaattcccgccgccatcgg
ccamk-1	Pair 2	ggtttctccatagtgagaagagg	gcgatgatggggatgcagcaggg
ccamk-2	Pair 1	gtcacagatgtcctcggccgagg	ggcttggccctgcgatgatgggg
cyclops-2	Pair 1	ggaggagttcatggagatggagg	gccgccgagcatggagatgatgg
cyclops-3	Pair 1	ggaggagttcatggagatggagg	gccgccgagcatggagatgatgg
ram1-1	Pair 1	ggagggacttcagccctctgagg	ggcaaagcctgaccagtgggcgg
ram1-2	Pair 2	ccaacctctacaacaacagcacc	gtcagtaatacagtcagatcagg
nsp1-1	Pair 1	ggagggacttcagccctctgagg	ggcaaagcctgaccagtgggcgg
nsp1-4	Pair 2	ccaacctctacaacaacagcacc	gtcagtaatacagtcagatcagg
nsp2-2	Pair 2	gtgggggatctcgagatctccgg	gcacgacgacgacctgccgcagg
nsp2-4	Pair 2	gtgggggatctcgagatctccgg	gcacgacgacgacctgccgcagg

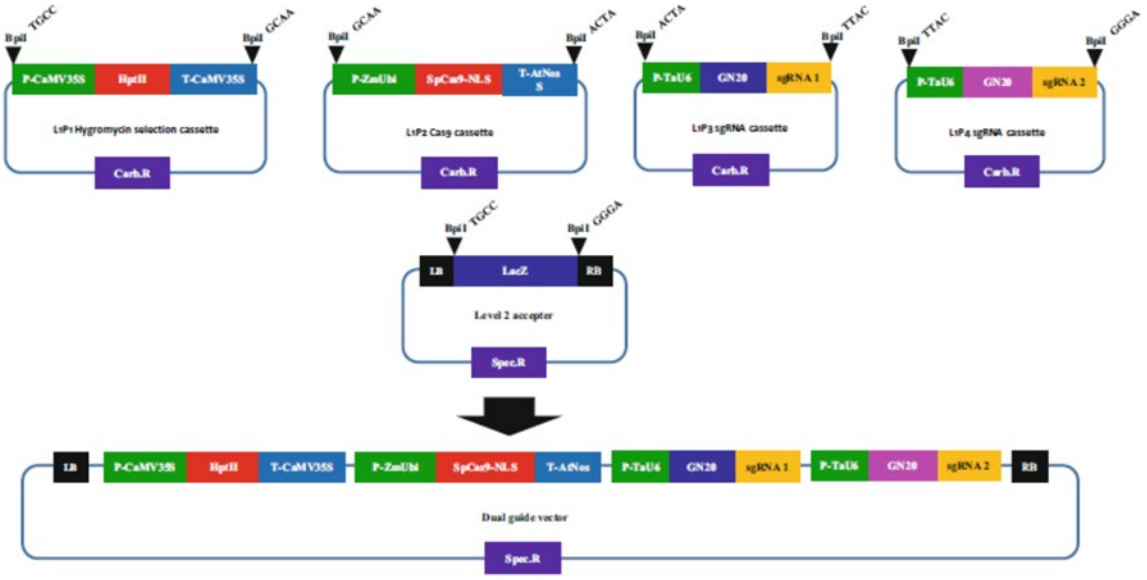


Figure 1. Schematic of Level 1 and Level 2 cassettes used in CRISPR/CAS9 editing system. Adapted from [11]. Level 1 cassettes are assembled into the level 2 accepter via BpiI cloning. Level 1 position 1 (L1P1) Hygromycin resistance cassette consisting of the hygromycin phosphotransferase coding sequence (hptII) driven and terminated by the 35 s promoter (P-CaMV35s). Level 1 position 2 (L1P2) Cas9 expression cassette consisting of sequence encoding Cas9 from *Streptococcus pyogenes* with a carboxy-terminal nuclearlocalization signal from Simian vacuolating virus 40 (SpCas9:NLS) driven by a ubiquitin promoter from Zea mays (P-ZmUbi) and terminated by a nopaline synthase terminator from *Agrobacterium tumefaciens* (T-AtNos). Level 1 position 3 and 4 (L1P3/L1P4) single guide RNA (sgRNA1 and sgRNA2) driven by a *Triticum aestivum* U6 promoter (P-TaU6). Selection of these plasmids in bacteria is carbenicillin (Level 1)) and spectinomycin (level 2). Left border (LB) and right border (RB) T-DNA sequences are indicated.

11. The nature and source of the vector used.

The plasmids for the production of the gene-edited lines and the gene modified lines were constructed using the Golden Gate cloning system [10, 13]. The gene sequences were domesticated and synthesized or PCR amplified, and then cloned into level 0 vector pMS or pMK (GeneArt, Thermo Fisher Scientific). The Golden Gate assembly cloning of the constructs to the level 1 and level 2 vectors were performed following standard protocols [14]. The Golden Gate cassette sequence is located between the two border sequences of the T-DNA region of the transformation vector. This enables the insertion of the genes to be transferred to plant hosts by the Golden Gate cloning technique [15]. This cloning cassette includes recognition sites for Type IIS restriction endonucleases and 4-nucleotide-overhang sequences to determine the polarity of the insert. The Golden Gate cassette includes the LacZ gene which is replaced by the genes of interest upon

cloning [15]. The orientation of the T-DNA boundary sequences in these vectors means that any new DNA integrated in the Golden Gate cassette site is transferred to the plant DNA. 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.

The schematic maps and information about genetic elemnts for the Level 2 constructs used to create gene-edited and gene-modified lines for this release can be found in Figures 2- 9 and Tables 2- 9.

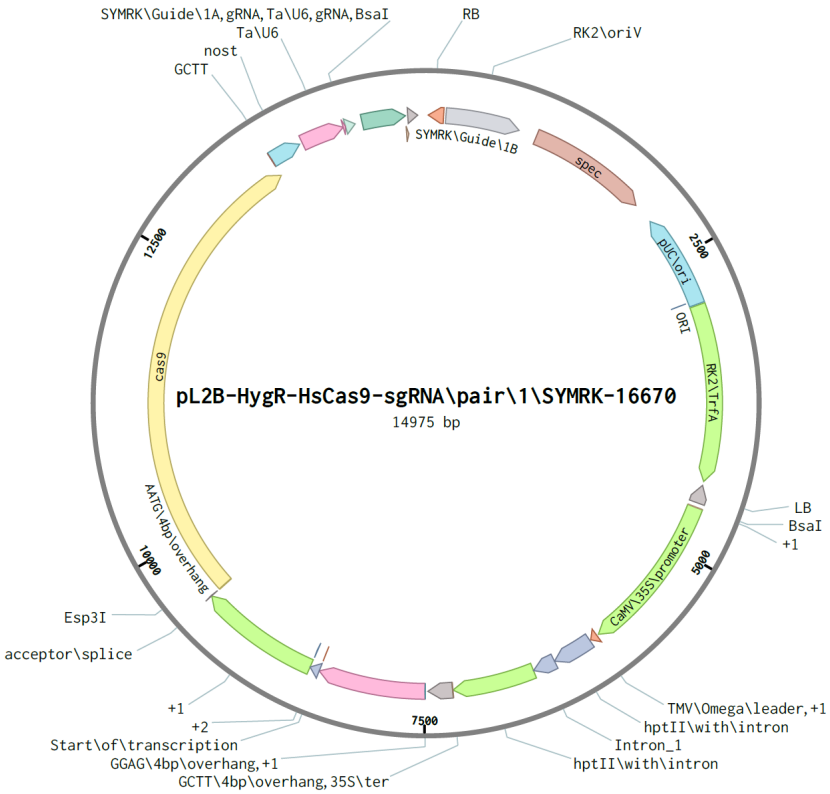


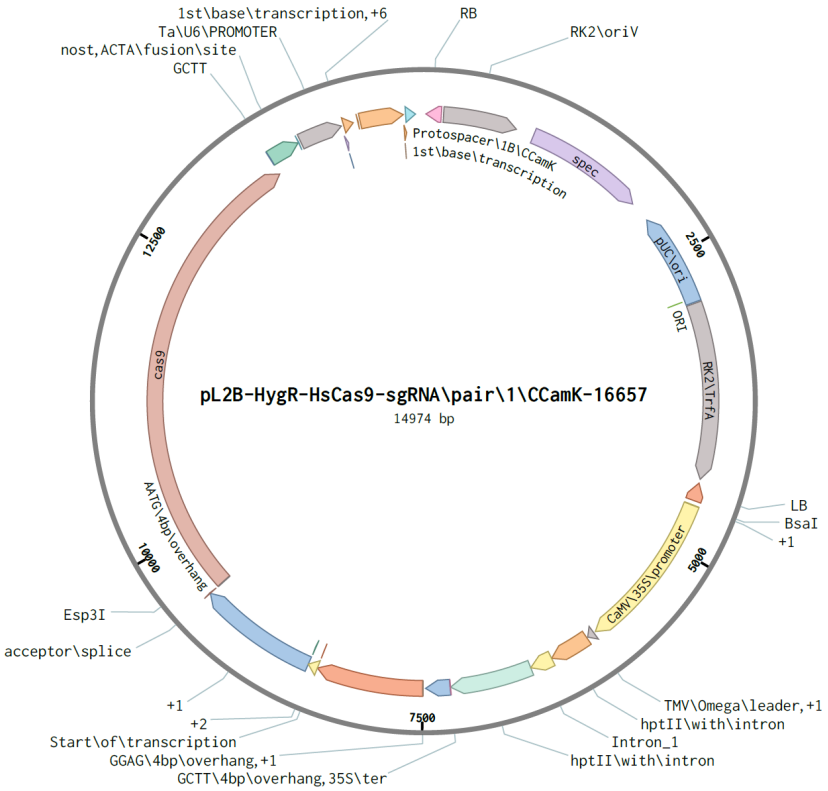
Figure 2. pL2B-HygR-HsCas9-sgRNApair1SYMRK. This plasmid contains two gRNA (1A and 1B) specific to *HvSYMRK* exonic regions under the control of wheat U6 gene promoter alongside of *Cas9* driven by a ubiquitin promoter from maize and terminated by a nopaline synthase terminator from *Agrobacterium tumefaciens* as well as hygromycin resistance cassette. The information about the fragments can be found in Table 2. Note that the *symrk-2* gene-edited line was developed using this plasmid.

Table 2. Genetic elements in the plasmid pL2B-HygR-HsCas9-sgRNApair1SYMRK.

Element	**Size (bp)**	**Donor Organism**	**Description and intend function**
RB	25	*Agrobacterium tumefaciens*	T-DNA right border
Spec^{R}	1008	*Escherichia coli*	Bacterial selection gene conferring resistance to Streptomycin.
pUC\Ori	790	*Escherichia coli*	Origin of replication for plasmid replication in *E. coli.*
LB	25	*Agrobacterium tumefaciens*	T-DNA right border

CaMV35S promoter	1338	Cauliflower mosaic virus (CaMV)	Promoter sequence from CaMV
hptII with intron	1216	*Escherichia coli* and Ricinus communis	Plant selectable marker gene encoding hygromycin phosphotransferase gene, including CAT-1 intron from Ricinus communis catalase-1 gene.
35S terminator	204	*Cauliflower Mosaic Virus (CaMV)*	gene terminator
Ubiquitin upstream promoter with intron	2001	*Zea mays*	Maize ubiquitin 1 (*Ubi1*) promoter + first intron driving constitutive expression
Cas9	4140	*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 with a carboxy-terminal nuclear localization signal from Simian vacuolating virus 40 (SpCas9:NLS)
Nos terminator	263	*Agrobacterium tumefaciens*	Nopaline Synthese Gene Terminator
pTaU6	363	*Triticum aestivum*	Wheat Sno U6 gene promotor driving constitutive expression
SYMRK\Guide\1A	20	*Hordeum vulgare*	20 nucleotides of the sgRNA complementary and unique to the exon 2 of HvSYMRK
SYMRK\Guide\1B	20	*Hordeum vulgare*	20 nucleotides of the sgRNA complementary and unique to the exon 3 of HvSYMRK
gRNA	83	*Streptococcus pyogenes*	The non-variable section of sgRNA

A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ORI
RK2\TrfA
LB
BsaI
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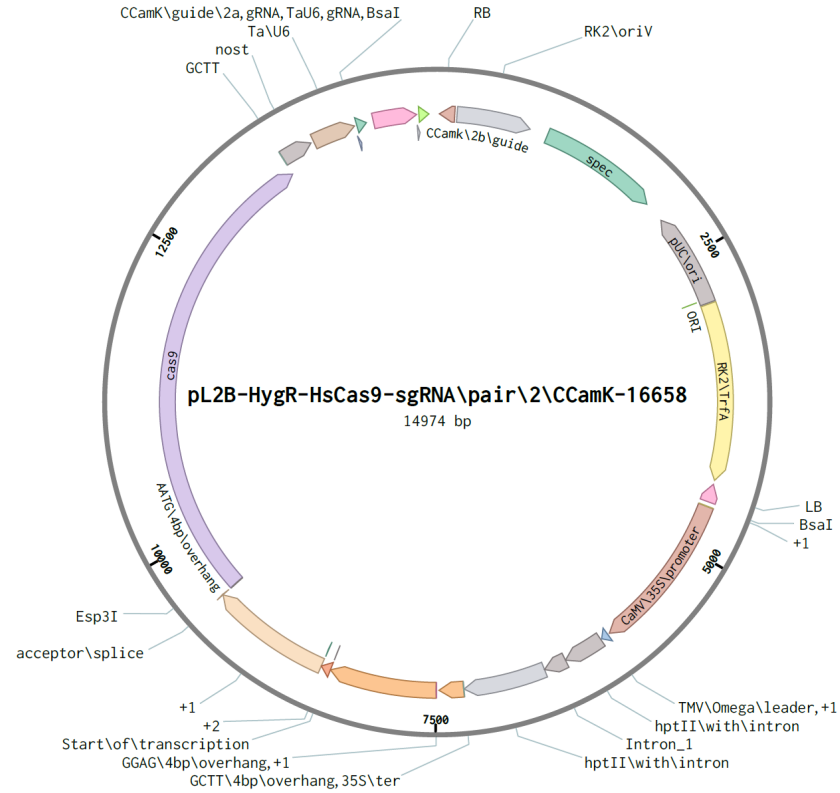
B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Intron_1
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Esp3I
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+2
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cas9
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5000
7500
10000
12500
pL2B-HygR-HsCas9-sgRNA\pair\2\CCamK-16658
14974 bp

Figure 3. pL2B-HygR-HsCas9-sgRNApair1CCamK **(A)** and pL2B-HygR-HsCas9-sgRNApair2CCamK **(B**). Each of these plasmids contains two gRNA (1A and 1B in plasmid **Figure 3A** and 2A and 2B in the plasmid **Figure 3B**) specific to *HvCCamK* exonic regions under the control of wheat U6 gene promoter alongside of *Cas9* driven by a ubiquitin promoter from maize and terminated by a nopaline synthase terminator from *Agrobacterium tumefaciens* as well as hygromycin resistance cassette. The information about the fragments can be found in Table 3. Note that the *ccamk-2* and *ccamk-1* gene-edited lines were developed using these plasmids, respectively.

Table 3. Genetic elements in the plasmid pL2B-HygR-HsCas9-sgRNApair1CCamK and plasmid pL2B-HygR-HsCas9-sgRNApair2CCamK.

Element	**Size (bp)**	**Donor Organism**	**Description and intend function**
RB	25	*Agrobacterium tumefaciens*	T-DNA right border
$Spec^R$	1008	*Escherichia coli*	Bacterial selection gene conferring resistance to Streptomycin.
pUC\Ori	790	*Escherichia coli*	Origin of replication for plasmid replication in *E. coli.*
LB	25	*Agrobacterium tumefaciens*	T-DNA right border
CaMV35S promoter	1338	Cauliflower mosaic virus (CaMV)	Promoter sequence from CaMV
hptII with intron	1216	*Escherichia coli* and Ricinus communis	Plant selectable marker gene encoding hygromycin phosphotransferase gene, including CAT-1 intron from Ricinus communis catalase-1 gene.
35S terminator	204	*Cauliflower Mosaic Virus (CaMV)*	gene terminator
Ubiquitin upstream promoter with intron	2001	*Zea mays*	Maize ubiquitin 1 (*Ubi1*) promoter + first intron driving constitutive expression

Cas9	4140	*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 with a carboxy-terminal nuclear localization signal from Simian vacuolating virus 40 (SpCas9:NLS)
Nos terminator	263	*Agrobacterium tumefaciens*	Nopaline Synthese Gene Terminator
pTaU6	363	*Triticum aestivum*	Wheat Sno U6 gene promotor driving constitutive expression
CCamK\Guide\1A	20	*Hordeum vulgare*	20 nucleotides of the sgRNA complementary and unique to the exon 1 of HvCCamK
CCamK\Guide\1B	20	*Hordeum vulgare*	20 nucleotides of the sgRNA complementary and unique to the exon 1 of HvCCamK
CCamK\Guide\2A	20	*Hordeum vulgare*	20 nucleotides of the sgRNA complementary and unique to the exon 1 of HvCCamK
CCamK\Guide\2B	20	*Hordeum vulgare*	20 nucleotides of the sgRNA complementary and unique to the exon 1 of HvCCamK
gRNA	83	*Streptococcus pyogenes*	The non-variable section of sgRNA

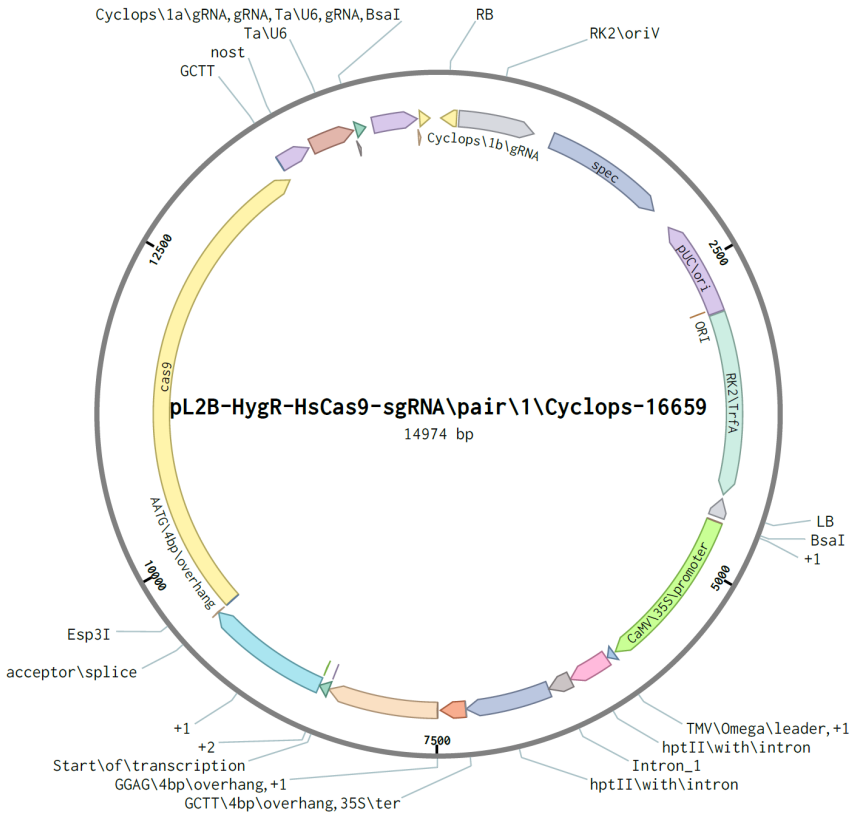


Figure 4. pL2B-HygR-HsCas9-sgRNApair1Cyclops. This plasmid contains two gRNA (1A and 1B) specific to *HvCyclops* exonic regions under the control of wheat U6 gene promoter alongside of *Cas9* driven by a ubiquitin promoter from maize and terminated by a nopaline synthase terminator from *Agrobacterium tumefaciens* as well as hygromycin resistance cassette. The information about the fragments can be found in Table 4. Note that the *cyclops-2* and cyclops-3 gene-edited lines were developed using this plasmid.

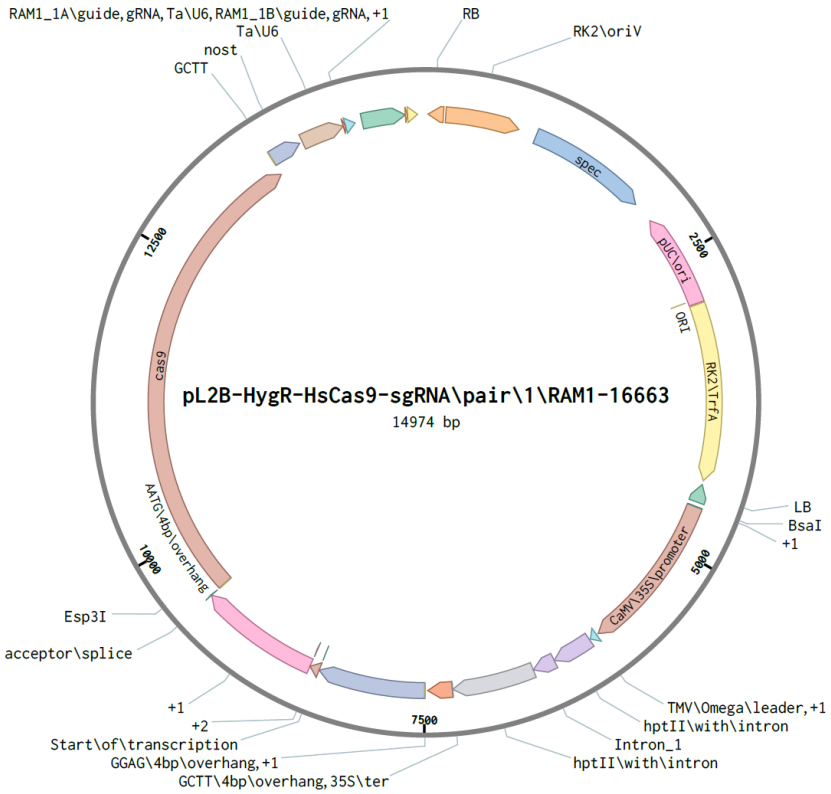
Table 4. Genetic elements in the plasmid pL2B-HygR-HsCas9-sgRNApair1Cyclops.

Element	**Size (bp)**	**Donor Organism**	**Description and intend function**
RB	25	*Agrobacterium tumefaciens*	T-DNA right border
$Spec^R$	1008	*Escherichia coli*	Bacterial selection gene conferring resistance to Streptomycin.
pUC\Ori	790	*Escherichia coli*	Origin of replication for plasmid replication in *E. coli.*

LB	25	*Agrobacterium tumefaciens*	T-DNA right border
CaMV35S promoter	1338	Cauliflower mosaic virus (CaMV)	Promoter sequence from CaMV
hptII with intron	1216	*Escherichia coli* and Ricinus communis	Plant selectable marker gene encoding hygromycin phosphotransferase gene, including CAT-1 intron from Ricinus communis catalase-1 gene.
35S terminator	204	*Cauliflower Mosaic Virus (CaMV)*	gene terminator
Ubiquitin upstream promoter with intron	2001	*Zea mays*	Maize ubiquitin 1 (*Ubi1*) promoter + first intron driving constitutive expression
Cas9	4140	*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 with a carboxy-terminal nuclear localization signal from Simian vacuolating virus 40 (SpCas9:NLS)
Nos terminator	263	*Agrobacterium tumefaciens*	Nopaline Synthese Gene Terminator
pTaU6	363	*Triticum aestivum*	Wheat Sno U6 gene promotor driving constitutive expression
CYCLOPS\Guide\1A	20	*Hordeum vulgare*	20 nucleotides of the sgRNA complementary and unique to the exon 1 of HvCyclops

CYCLOPS\Guide\1B	20	*Hordeum vulgare*	20 nucleotides of the sgRNA complementary and unique to the exon 1 of HvCyclops
gRNA	83	*Streptococcus pyogenes*	The non-variable section of sgRNA

A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B

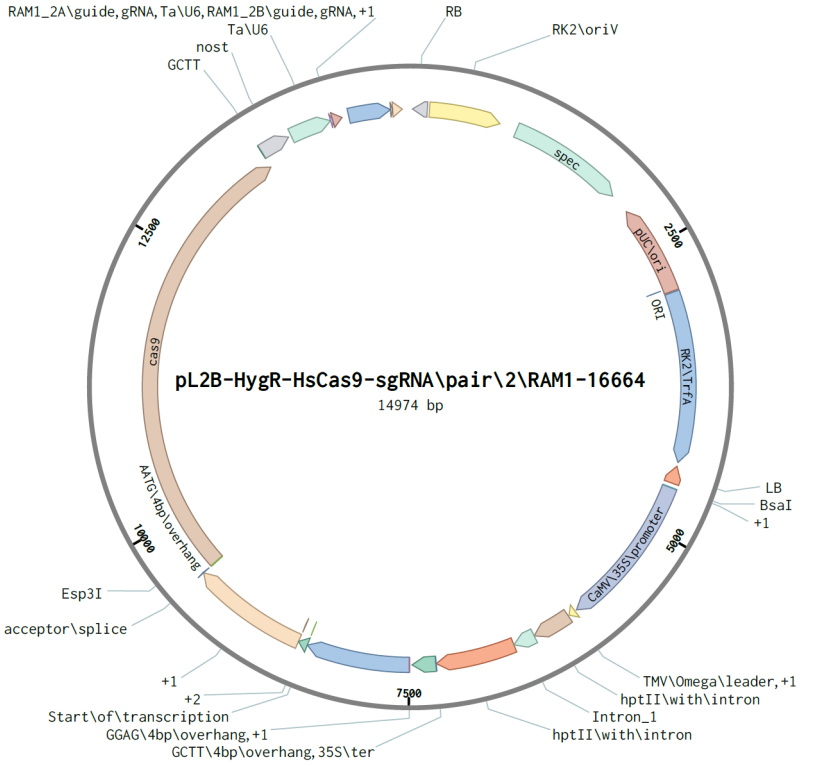

RAM1_2A\guide, gRNA, Ta\U6, RAM1_2B\guide, gRNA, +1
Ta\U6
nost
GCTT
RB
RK2\oriV
spec
pUC\ori
ORI
RK2\TrfA
LB
BsaI
+1
CaMV\35S\promoter
TMV\Omega\leader, +1
hptII\with\intron
Intron_1
hptII\with\intron
GCTT\4bp\overhang, 35S\ter
GGAG\4bp\overhang, +1
Start\of\transcription
+2
+1
acceptor\splice
Esp3I
AATG\4bp\overhang
cas9
pL2B-HygR-HsCas9-sgRNA\pair\2\RAM1-16664
14974 bp
2500
5000
7500
10000
12500

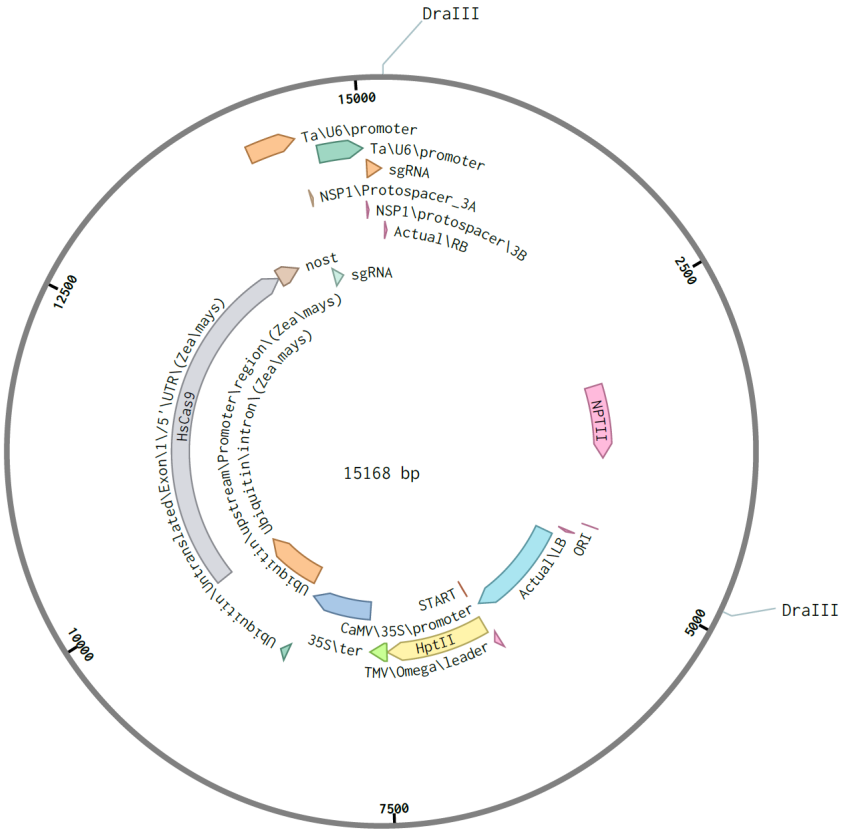
Figure 5. pL2B-HygR-HsCas9-sgRNApair1RAM1 **(A)** and pL2B-HygR-HsCas9-sgRNApair2RAM1 **(B**). Each of these plasmids contains two gRNA (1A and 1B in plasmid **Figure 5A** and 2A and 2B in the plasmid **Figure 5B**) specific to *HvRAM1* exonic regions under the control of wheat U6 gene promoter alongside of *Cas9* driven by a ubiquitin promoter from maize and terminated by a nopaline synthase terminator from *Agrobacterium tumefaciens* as well as hygromycin resistance cassette. The information about the fragments can be found in Table 5. Note that the *ram1-1* and *ram1-2* gene-edited lines were developed using these plasmids, respectively.

Table 5. Genetic elements in the plasmid pL2B-HygR-HsCas9-sgRNApair1RAM1 and plasmid pL2B-HygR-HsCas9-sgRNApair2RAM1.

Element	**Size (bp)**	**Donor Organism**	**Description and intend function**
RB	25	*Agrobacterium tumefaciens*	T-DNA right border
$Spec^R$	1008	*Escherichia coli*	Bacterial selection gene conferring resistance to Streptomycin.
pUC\Ori	790	*Escherichia coli*	Origin of replication for plasmid replication in *E. coli.*
LB	25	*Agrobacterium tumefaciens*	T-DNA right border
CaMV35S promoter	1338	Cauliflower mosaic virus (CaMV)	Promoter sequence from CaMV
hptII with intron	1216	*Escherichia coli* and Ricinus communis	Plant selectable marker gene encoding hygromycin phosphotransferase gene, including CAT-1 intron from Ricinus communis catalase-1 gene.
35S terminator	204	*Cauliflower Mosaic Virus (CaMV)*	gene terminator
Ubiquitin upstream promoter with intron	2001	*Zea mays*	Maize ubiquitin 1 (*Ubi1*) promoter + first intron driving constitutive expression

Cas9	4140	*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 with a carboxy-terminal nuclear localization signal from Simian vacuolating virus 40 (SpCas9:NLS)
Nos terminator	263	*Agrobacterium tumefaciens*	Nopaline Synthese Gene Terminator
pTaU6	363	*Triticum aestivum*	Wheat Sno U6 gene promotor driving constitutive expression
RAM1\Guide\1A	20	*Hordeum vulgare*	20 nucleotides of the sgRNA complementary and unique to the exon 1 of HvRAM1
RAM1\Guide\1B	20	*Hordeum vulgare*	20 nucleotides of the sgRNA complementary and unique to the exon 1 of HvRAM1
RAM1\Guide\2A	20	*Hordeum vulgare*	20 nucleotides of the sgRNA complementary and unique to the exon 1 of HvRAM1
RAM1\Guide\2B	20	*Hordeum vulgare*	20 nucleotides of the sgRNA complementary and unique to the exon 1 of HvRAM1
gRNA	83	*Streptococcus pyogenes*	The non-variable section of sgRNA

A


DraIII
15000
Ta\U6\promoter
Ta\U6\promoter
sgRNA
NSP1\Protospacer_3A
NSP1\protospacer\3B
Actual\RB
2500
nost
sgRNA
12500
Ubiquitin\intron\(Zea\mays)
Ubiquitin\upstream\Promoter\region\(Zea\mays)
HsCas9
Ubiquitin\untranslated\Exon\1\5'\UTR\(Zea\mays)
15168 bp
NPTII
Actual\LB
ORI
START
CaMV\35S\promoter
HptII
35S\ter
TMV\Omega\leader
10000
5000
DraIII
7500

B

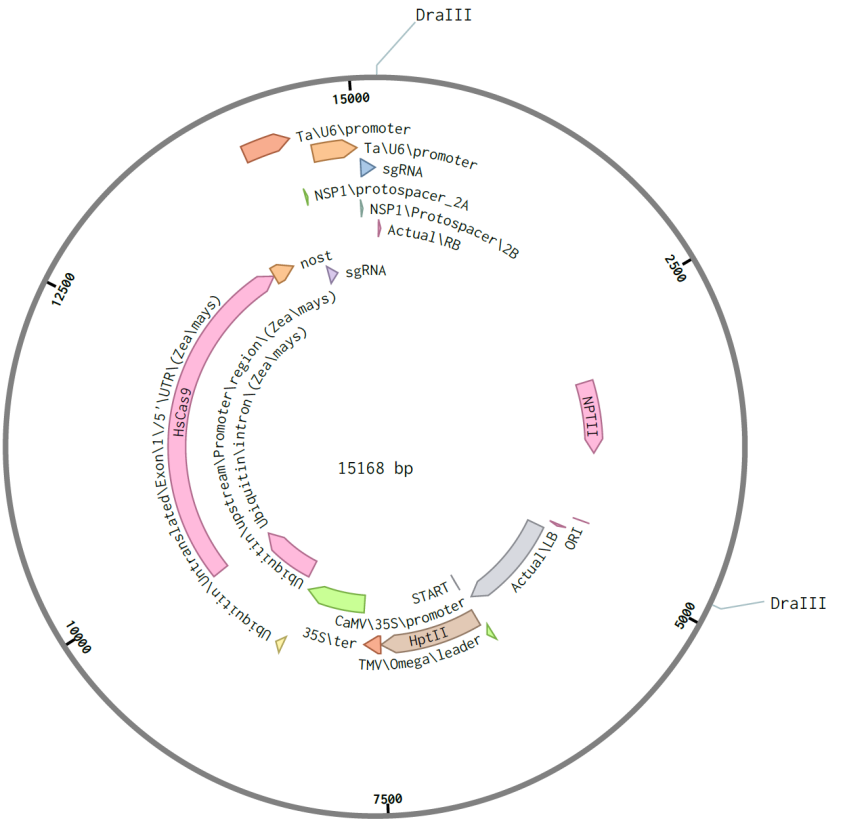

DraIII
15000
Ta\U6\promoter
Ta\U6\promoter
sgRNA
NSP1\protospacer_2A
NSP1\Protospacer\2B
Actual\RB
2500
nost
sgRNA
12500
Ubiquitin\intron\(Zea\mays)
Ubiquitin\upstream\Promoter\region\(Zea\mays)
HsCas9
Ubiquitin\untranslated\Exon\1\5'\UTR\(Zea\mays)
15168 bp
NPTII
Actual\LB
ORI
START
CaMV\35S\promoter
HptII
35S\ter
TMV\Omega\leader
10000
5000
DraIII
7500

Figure 6. pL2B-HygR-HsCas9-sgRNApair1NSP1 **(A)** and pL2B-HygR-HsCas9-sgRNApair2NSP1 **(B**). Each of these plasmids contains two gRNA (1A and 1B in plasmid **Figure 6A** and 2A and 2B in the plasmid **Figure 6B**) specific to *HvNSP1* exonic regions under the control of wheat U6 gene promoter alongside of *Cas9* driven by a ubiquitin promoter from maize and terminated by a nopaline synthase terminator from *Agrobacterium tumefaciens* as well as hygromycin resistance cassette. The information about the fragments can be found in Table 6. Note that the *nsp1-1* and *nsp1-4* gene-edited lines were developed using these plasmids, respectively.

Table 6. Genetic elements in the plasmid pL2B-HygR-HsCas9-sgRNApair1NSP1 and plasmid pL2B-HygR-HsCas9-sgRNApair2NSP1.

Element	**Size (bp)**	**Donor Organism**	**Description and intend function**
RB	25	*Agrobacterium tumefaciens*	T-DNA right border
$Spec^R$	1008	*Escherichia coli*	Bacterial selection gene conferring resistance to Streptomycin.
pUC\Ori	790	*Escherichia coli*	Origin of replication for plasmid replication in *E. coli.*
LB	25	*Agrobacterium tumefaciens*	T-DNA right border
CaMV35S promoter	1338	Cauliflower mosaic virus (CaMV)	Promoter sequence from CaMV
hptII with intron	1216	*Escherichia coli* and Ricinus communis	Plant selectable marker gene encoding hygromycin phosphotransferase gene, including CAT-1 intron from Ricinus communis catalase-1 gene.
35S terminator	204	*Cauliflower Mosaic Virus (CaMV)*	gene terminator
Ubiquitin upstream promoter with intron	2001	*Zea mays*	Maize ubiquitin 1 (*Ubi1*) promoter + first intron driving constitutive expression

Cas9	4140	*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 with a carboxy-terminal nuclear localization signal from Simian vacuolating virus 40 (SpCas9:NLS)
Nos terminator	263	*Agrobacterium tumefaciens*	Nopaline Synthese Gene Terminator
pTaU6	363	*Triticum aestivum*	Wheat Sno U6 gene promotor driving constitutive expression
NSP1Guide\1A	20	*Hordeum vulgare*	20 nucleotides of the sgRNA complementary and unique to the single exon of HvNSP1
NSP1\Guide\1B	20	*Hordeum vulgare*	20 nucleotides of the sgRNA complementary and unique to the single exon of HvNSP1
NSP1\Guide\2A	20	*Hordeum vulgare*	20 nucleotides of the sgRNA complementary and unique to the single exon of HvNSP1
NSP1\Guide\2B	20	*Hordeum vulgare*	20 nucleotides of the sgRNA complementary and unique to the single exon of HvNSP1
gRNA	83	*Streptococcus pyogenes*	The non-variable section of sgRNA

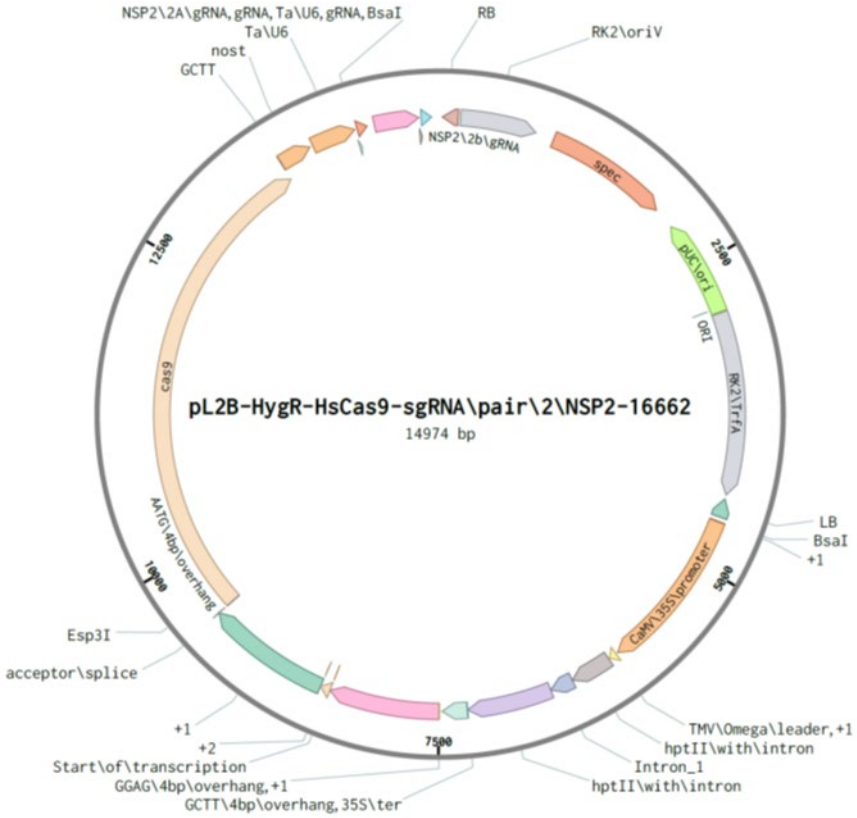


Figure 7. pL2B-HygR-HsCas9-sgRNApair1NSP2. This plasmid contains two gRNA (1A and 1B) specific to *HvNSP2* exonic regions under the control of wheat U6 gene promoter alongside of *Cas9* driven by a ubiquitin promoter from maize and terminated by a nopaline synthase terminator from *Agrobacterium tumefaciens* as well as hygromycin resistance cassette. The information about the fragments can be found in Table 7. Note that the *nsp2-2* and *nsp2-4* gene-edited lines were developed using this plasmid.

Table 7. Genetic elements in the plasmid pL2B-HygR-HsCas9-sgRNApair1NSP2.

Element	**Size (bp)**	**Donor Organism**	**Description and intend function**
RB	25	*Agrobacterium tumefaciens*	T-DNA right border
Spec^{R}	1008	*Escherichia coli*	Bacterial selection gene conferring resistance to Streptomycin.
pUC\Ori	790	*Escherichia coli*	Origin of replication for plasmid replication in *E. coli.*
LB	25	*Agrobacterium tumefaciens*	T-DNA right border

CaMV35S promoter	1338	Cauliflower mosaic virus (CaMV)	Promoter sequence from CaMV
hptII with intron	1216	*Escherichia coli* and Ricinus communis	Plant selectable marker gene encoding hygromycin phosphotransferase gene, including CAT-1 intron from Ricinus communis catalase-1 gene.
35S terminator	204	*Cauliflower Mosaic Virus (CaMV)*	gene terminator
Ubiquitin upstream promoter with intron	2001	*Zea mays*	Maize ubiquitin 1 (*Ubi1*) promoter + first intron driving constitutive expression
Cas9	4140	*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 with a carboxy-terminal nuclear localization signal from Simian vacuolating virus 40 (SpCas9:NLS)
Nos terminator	263	*Agrobacterium tumefaciens*	Nopaline Synthese Gene Terminator
pTaU6	363	*Triticum aestivum*	Wheat Sno U6 gene promotor driving constitutive expression
NSP2\Guide\2A	20	*Hordeum vulgare*	20 nucleotides of the sgRNA complementary and unique to the single exon of HvNSP2s
NSP2\Guide\2B	20	*Hordeum vulgare*	20 nucleotides of the sgRNA complementary and unique to the single exon of HvNSP2s
gRNA	83	*Streptococcus pyogenes*	The non-variable section of sgRNA

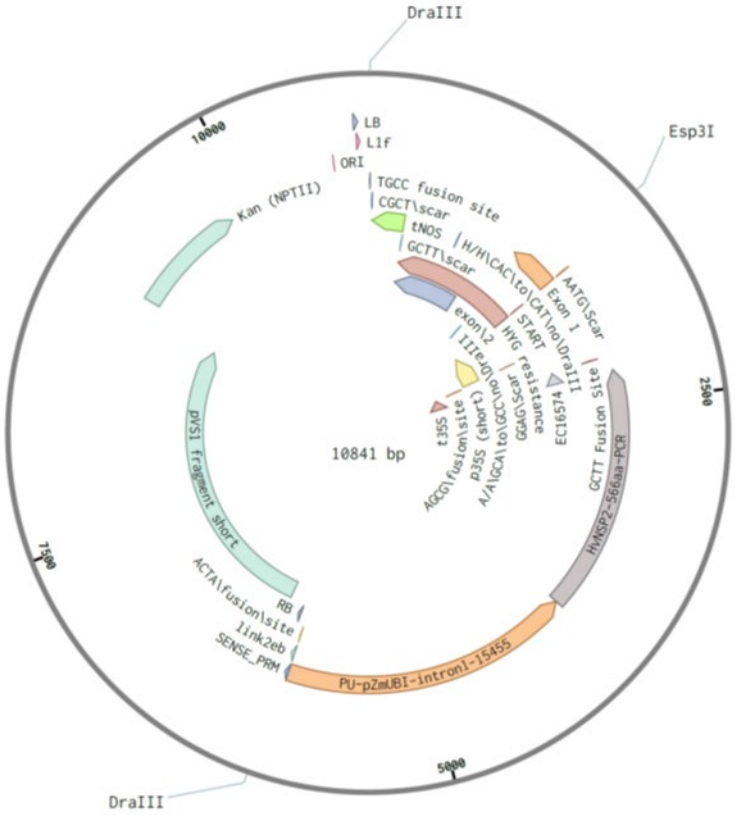


Figure 8. pL2B-HYG-pZmUBI-HvNSP2-3xMyc. This plasmid contains *HvNSP2* fused to myc-tag under maize ubiquitin and terminated by a nopaline synthase terminator from *Agrobacterium tumefaciens* as well as hygromycin resistance cassette. The information about the fragments can be found in Table 8. Note that the OxHvNSP2 overexpression gene-modified lines was developed using this plasmid.

Table 8. Genetic elements in the plasmid pL2B-HYG-pZmUBI-HvNSP2-3xMyc.

Element	**Size (bp)**	**Donor Organism**	**Description and intend function**
RB	25	*Agrobacterium tumefaciens*	T-DNA right border
Kan (NPTII)	795	*Escherichia coli*	Neomycin Phosphotransferase II gene, Bacterial selection gene conferring resistance to kanamycin.
pUC\Ori	790	*Escherichia coli*	Origin of replication for plasmid replication in *E. coli.*
LB	25	*Agrobacterium tumefaciens*	T-DNA right border

CaMV35S promoter short	425	Cauliflower mosaic virus (CaMV)	Short version of Promoter sequence from CaMV
hptII with intron	1216	*Escherichia coli* and Ricinus communis	Plant selectable marker gene encoding hygromycin phosphotransferase gene, including CAT-1 intron from Ricinus communis catalase-1 gene.
Nos terminator	263	*Agrobacterium tumefaciens*	Nopaline Synthese Gene Terminator
Ubiquitin upstream promoter with intron	2001	*Zea mays*	Maize ubiquitin 1 (*Ubi1*) promoter + first intron driving constitutive expression
HvNSP2	1701	*Hordeum vulgare*	Encodes the Nodulation Signalling Pathway 2 (NSP2) from barley, *HvNSP2* - a GRAS domain transcriptional regulator, which is an ERF transcription factor
3 X Myc-tag	100	*human c-Myc*	A polypeptide epitope protein tag (EQKLISEEDL) translationally fused to HvNSP2, used for affinity chromatography of the overexpressed recombinant protein
35S terminator	204	*Cauliflower Mosaic Virus (CaMV)*	gene terminator

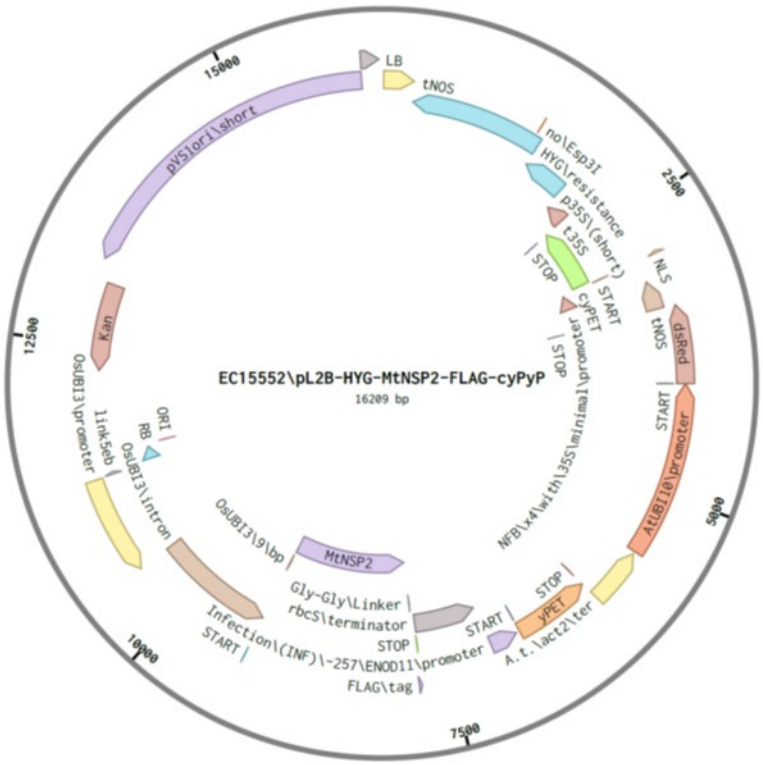


Figure 9. pL2B-HYG-pZmUBI-MtNSP2-FLAG-CyPyP. This plasmid contains MtNSP2 fused to Flag-tag under rice ubiquitin promoter and terminated by a terminator of ribulose-1,5-bisphosphate carboxylase gene from *Chrysanthemum morifolium* as well as hygromycin resistance cassette and three reporter genes. The information about the fragments can be found in Table 9. Note that the OxMtNSP2 overexpression gene-modified lines was developed using this plasmid.

Table 9. Genetic elements in the plasmid pL2B-HYG-pZmUBI-MtNSP2-FLAG-CyPyP.

Element	**Size (bp)**	**Donor Organism**	**Description and intend function**
RB	25	*Agrobacterium tumefaciens*	T-DNA right border
Kan (NPTII)	795	*Escherichia coli*	Neomycin Phosphotransferase II gene, Bacterial selection gene conferring resistance to kanamycin.
pUC\Ori	790	*Escherichia coli*	Origin of replication for plasmid replication in *E. coli.*

LB	25	*Agrobacterium tumefaciens*	T-DNA right border
CaMV35S promoter short	425	Cauliflower mosaic virus (CaMV)	Short version of Promoter sequence from CaMV
hptII with intron	1216	*Escherichia coli* and Ricinus communis	Plant selectable marker gene encoding hygromycin phosphotransferase gene, including CAT-1 intron from Ricinus communis catalase-1 gene.
Nos terminator	263	*Agrobacterium tumefaciens*	Nopaline Synthese Gene Terminator
Ubiquitin upstream promoter with intron	1149	*Oryza sativa*	Rice ubiquitin 3 (*Ubi3*) promoter + first intron driving constitutive expression
MtNSP2	1524	*Medicago truncatula*	Encodes the Nodulation Signalling Pathway 2 (NSP2) from *Medicago truncatula*, *MtNSP2*- a GRAS domain transcriptional regulator, which is an ERF transcription factor
FLAG-tag	27	*Artificial design*	A polypeptide epitope protein tag (DYKDDDK) translationally fused to MtNSP2, used for affinity chromatography of the overexpressed recombinant protein
rbcS terminator	647	*Chrysanthemum morifolium*	Terminator of ribulose-1,5-bisphosphate carboxylase gene
pINF (promoter)	257	*Medicago truncatula*	Rhizobia Infection region of *ENDO11* promoter + minimal promoter
yPET	717	Originally derived from *Aequorea victoria.*	Encodes for Yellow fluorescent protein
tAct2	485	*Arabidopsis thaliana*	Terminator Actin 2 gene
AtUBI10 promoter	1511	*Arabidopsis thaliana*	polyubiquitin 10 promoter and intron;

dsRED	678	Originally from *Discosoma*	Encodes a fluorophore that fluoresces red-orange when excited
Nos terminator	263	*Agrobacterium tumefaciens*	Nopaline Synthese Gene Terminator
pNFP (promoter)	178	*Medicago truncatula*	4 X NF box of *ENOD11* promoter + minimal promoter
cyPET	717	Originally derived from *Aequorea victoria.*	Encodes a fluorophore which fluoresces cyan
35S terminator	204	*Cauliflower Mosaic Virus (CaMV)*	35S terminator

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 aim of this study is to evaluate the impact on biomass and yield following the use of commercial arbuscular mycorrhizal fungi (AMF) mixed inoculum on the performance of the gene-edited and genetically modified barley cv. Golden Promise lines. The CRISPR mutant gene-edited lines used in this release have aborted or significantly reduced colonization by AMF. The *symrk-2* gene-edited line lacks the Symbiosis Receptor-like kinase (SYMRK) which in wild-type plants allows recognition of AM fungi and is essential for mycorrhizal colonisation. As a result, *symrk* mutants are unable to form mycorrhizal symbioses. In wild-type plants, perception of the signalling molecules from fungi by receptor-like kinases triggers nuclear calcium spiking. A nuclear-localized calcium and calmodulin-dependent kinase (CCaMK) interacts with and phosphorylates the transcription factor CYCLOPS. CCaMK and CYCLOPS are required AMF colonisation, therefore, *ccamk* and *cyclops* mutant lines (i.e., *ccamk-1*, *ccamk-2*, *cyclops-2*, and *cyclops-3* gene-edited lines) are unable to get colonized by fungi. *REDUCED ARBUSCULAR MYCORRHIZA1* (*RAM1*) acts downstream of the CCaMK-CYCLOPS complex and is required for arbuscule development, with mutations in *ram1* (for instance, in *ram1-1* and *ram1-2* gene-edited lines) lead to abortion of AM interactions. The *nsp1* and *nsp2* gene-edited mutant lines (*nsp1-1*, *nsp1-4*, *nsp2-2*, and *nsp2-4*) show reduced production of strigolactones, molecules that promote mycorrhizal fungal spore formation and subsequent root colonisation by AMF. The *nsp* mutants have reduced mycorrhizal colonisation as a result. Conversely, the *NSP2* genetically modified overexpression lines (OxHvNSP2 and OXMtNSP2) show higher mycorrhizal colonisation, even at high soil phosphorus concentrations which usually act to suppress mycorrhization.

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

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

(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 information about the nature of the insertion and/or deletion, their length and their positions, and also the method for their selection are shown in Table 10. The T0 plants that contain only one copy of the insertion were taken forward, and those selected that possessed gene edited events at the relevant site. Lines carrying the gene edits and lacking the transgene were selected. Hence, there is no transgene in any of the CRISPR gene-edited lines, even in the first year of the trial. The insertions in the overexpression lines are expected to be chromosomal. Note that OxHvNSP2 lines were developed in the *nsp2-2* gene-edited background.

Table 10. Information about the inserted and deleted sequence.

Line description	Genetic editions	Genetic modifications	Characterization methods	Copy number	Method to identify transgene copy number
symrk-2	a 2 bp deletion at + 94-95 bp and a 31 bp deletion from +719 bp to +749 bp	N/A (Transgene free)	PCR with gene specific primers flanking the designed gRNAs followed by Sanger sequencing	Homozygous for the mutation	Taqman qPCR assay for HptII
ccamk-1	a 4 bp deletion from +166 bp to +169 bp	N/A (Transgene free)	PCR with gene specific primers flanking the designed gRNAs followed by Sanger sequencing	Homozygous for the mutation	Taqman qPCR assay for HptII
ccamk-2	a 1 bp deletion at +58 bp	N/A (Transgene free)	PCR with gene specific primers flanking the designed gRNAs followed by Sanger sequencing	Homozygous for the mutation	Taqman qPCR assay for HptII
cyclops-2	a 1 bp deletion at +8 bp and a 35 bp deletion from +113 bp	N/A (Transgene free)	PCR with gene specific primers flanking the designed gRNAs followed by Sanger sequencing	Homozygous for the mutation	Taqman qPCR assay for HptII

	to +147 bp				
cyclops-3	a 4 bp deletion from +8 bp to +11 bp and a 2bp deletion at +114-115 bp	N/A (Transgene free)	PCR with gene specific primers flanking the designed gRNAs followed by Sanger sequencing	Homozygous for the mutation	Taqman qPCR assay for HptII
ram1-1	a 1 bp insertion at +961 bp	N/A (Transgene free)	PCR with gene specific primers flanking the designed gRNAs followed by Sanger sequencing	Homozygous for the mutation	Taqman qPCR assay for HptII
ram1-2	a 1 bp insertion at +1013 bp	N/A (Transgene free)	PCR with gene specific primers flanking the designed gRNAs followed by Sanger sequencing	Homozygous for the mutation	Taqman qPCR assay for HptII
nsp1-1	a 1 bp deletion at +164 bp	N/A (Transgene free)	PCR with gene specific primers flanking the designed gRNAs followed by Sanger sequencing	Homozygous for the mutation	Taqman qPCR assay for HptII
nsp1-4	a 4 bp deletion from +286 bp to +289 bp	N/A (Transgene free)	PCR with gene specific primers flanking the designed gRNAs followed by Sanger sequencing	Homozygous for the mutation	Taqman qPCR assay for HptII
nsp2-2	a 314 bp deletion from +48 bp to +361 bp	N/A (Transgene free)	PCR with gene specific primers flanking the designed gRNAs followed by Sanger sequencing	Homozygous for the mutations	Taqman qPCR assay for HptII
nsp2-4	a 3 bp deletion from +38 bp to +40	N/A (Transgene free)	PCR with gene specific primers flanking the designed gRNAs followed by	Homozygous for the mutations	Taqman qPCR assay for HptII

	bp and a 1 bp insertion at +356 bp		Sanger sequencing		
OxHvNSP2	a 314 bp deletion from +48 bp to +361 bp (*nsp2-2*)	6137 bp insertion	PCR with insert specific primers followed by Sanger sequencing	Homozygous for one copy of the insertion	Taqman qPCR assay for HptII
OxMtNSP2	N/A	11435 bp insertion	PCR with insert specific primers followed by Sanger sequencing	Homozygous for one copy of the insertion	Taqman qPCR assay for HptII

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.

Agrobacterium-mediated transformation generates stable insertions. So far, no genetic or phenotypic instability has been observed in the lines that have been tested in the glasshouse. The plants that will be taken to the field if the permit is granted, have been propagated from the gene-edited and genetically modified plant lines which have been genetically and phenotypically well-characterized and have shown consistent results. Only lines for which no changes in genotype and phenotype are evident will be selected for field release.

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

The gene-edited plants used in the field trial are considered to be transgene free and free of CAS9 editing machinery system. The T1 parental lines were tested for the absence of *Cas9* gene and *Hyg* selectable marker gene. T1 plants which contain no T-DNA but have been observed to contain targeted mutations were taken forward. Therefore, further editing cannot occur in subsequent generations and/or in nature. Moreover, two independent genetically modified overexpression lines will be used in this release; the *HvNSP2* coding sequence fused to myc-tag expressed under the constitutive expression of maize ubiquitin promoter alongside hygromycin selectable marker, and the *MtNSP2* coding sequence fused to FLAG-tag expressed under the constitutive expression of the rice ubiquitin promoter alongside the hygromycin selectable marker and three other readout marker gene cassettes. The information for transgenic generations of genetically edited lines will be used in this release is summarized in Table 11.

Table 11. Information about transgenic generation of the lines used for the first year of the release.

Line number	Species	Cultivar	Line description	Transgenic generation for the first year of release
1	*Hordeum vulgare*	Golden Promise	*symrk-2*	T4
2	*Hordeum vulgare*	Golden Promise	*ccamk-1*	T5

3	*Hordeum vulgare*	Golden Promise	*ccamk-2*	T5
4	*Hordeum vulgare*	Golden Promise	*cyclops-2*	T4
5	*Hordeum vulgare*	Golden Promise	*cyclops-3*	T3
6	*Hordeum vulgare*	Golden Promise	*ram1-1*	T4
7	*Hordeum vulgare*	Golden Promise	*ram1-2*	T4
8	*Hordeum vulgare*	Golden Promise	*nsp1-1*	T3
9	*Hordeum vulgare*	Golden Promise	*nsp1-4*	T3
10	*Hordeum vulgare*	Golden Promise	*nsp2-2*	T5
11	*Hordeum vulgare*	Golden Promise	*nsp2-4*	T5
12	*Hordeum vulgare*	Golden Promise	OxHvNSP2	T2
13	*Hordeum vulgare*	Golden Promise	OxMtNSP2	T3

Part IVA Information on specific areas of risk

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

The phenotype of the gene-edited and genetically modified barley lines in this field trial, including morphology, pollination, and seed-set do not appear to differ from wild-type barley cv. Golden Promise plants. We therefore expect no difference in the dissemination of pollen and seeds compared to wild-type barley plants. There is no evidence of any change in the plants' ability to transfer genetic material to sexually compatible relatives since there are no reports of wild barley (*Hordeum spontaneum)* in the UK with which the lines used in this release could outcross.

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

The plasmids that were used through the transformation process leading to the gene-edited lines possess a bacterial origin of replication and antibiotic resistance marker genes for bacterial selection, but these components were not transferred to plants. Such plasmids were used to create the T0 plant generation, and their seeds utilized for further work. In the T1 generation of gene-edited lines, we have selected for lines which contain no T-DNA but have been observed to contain targeted mutations, classified as transgene-free mutants, and therefore free of CAS9 editing machinery. The plasmids used in the *Agrobacterium*-mediated transformation process do not exist even in the T2 plant lines. For the information about the transgenic generation of the lines which will be used in the first year of the release, refer to Table 11.

For genetically modified overexpression lines in this field trial (OxHvNSP2 and OXMtNSP2), it is reasonable to assume that the elements carried by the vector 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 (i.e., Hygromycin). However, we estimate the probability that horizontal gene transfer could occur to be extremely low. It is worth noting that there is no origin for bacterial replication within the DNA transferred to the plant. 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,

The target organisms of this field trial are the naturally occurring arbuscular mycorrhizal fungi. The aim of this study is to evaluate the impact on biomass and yield following the use of commercial arbuscular mycorrhizal fungi (AMF) mixed inoculum on the performance of the gene-edited and genetically modified barley cv. Golden Promise lines. The CRISPR mutant gene-edited lines used in this release have aborted or significantly reduced colonization by AMF. The *symrk-2* gene-edited line lacks the Symbiosis Receptor-like kinase (SYMRK) which in wild-type plants allows recognition of AM fungi and is essential for mycorrhizal colonisation. As a result, *symrk* mutants are unable to form mycorrhizal symbioses. In wild-type Golden Promise plants, perception of the signalling molecules from fungi by receptor-like kinases triggers nuclear calcium spiking. A nuclear-localized calcium and calmodulin-dependent kinase (CCaMK) interacts with and phosphorylates the transcription factor CYCLOPS. CCaMK and CYCLOPS are required for AMF colonisation. Therefore, *ccamk* and *cyclops* mutant lines (i.e., *ccamk-1*, *ccamk-2*, *cyclops-2*, and *cyclops-3* gene-edited lines) are unable to get colonized by fungi. *REDUCED ARBUSCULAR MYCORRHIZA1* (*RAM1*) acts downstream of the CCaMK-CYCLOPS complex and is required for arbuscule development, with mutations in *ram1* (for instance, in *ram1-1* and *ram1-2* gene-edited lines) lead to abortion of AM interactions. The *nsp1* and *nsp2* gene-edited mutant lines (*nsp1-1*, *nsp1-4*, *nsp2-2*, and *nsp2-4*) show reduced production of strigolactones, molecules that promote mycorrhizal fungal spore formation and subsequent root colonisation by AMF. The *nsp* mutants have reduced mycorrhizal colonisation as a result. Conversely, the *NSP2* genetically modified overexpressing lines show higher mycorrhizal colonisation, even at high soil phosphorus concentrations which usually act to suppress mycorrhization. Plots containing *NSP2* overexpression lines (OxHvNSP2 and OxMtNSP2) may show a slightly increased extent of soil-borne mycorrhizal fungal mycelium, while plots containing the genetically edited line including *symrk-2*, *ccamk-1*, *ccamk-2*, *cyclops-2*, *cyclops-3*, *ram1-1*, *ram1-2*, *nsp1-1*, *nsp1-4*, *nsp2-2, and nsp2-4* will likely have reduced quantities of these fungi. Changes in the extent of mycorrhizal colonisation and soil mycorrhizal hyphae in genetically modified and gene-edited lines are not expected to have any adverse environmental effect over the time span of this trial or thereafter, and will not affect the subsequent ability of mycorrhizal interactions to form in these soils.

19. Potential changes in the interactions of the genetically modified plant with no-target organisms resulting from the genetic modification and the adverse environmental effects arising,

Barley interacts with a range of pests and fungal pathogens and may also interact with multiple fungi, bacteria, and protists in the rhizosphere. Mycorrhizal fungal colonisation has been demonstrated to reduce plant susceptibility to pests and pathogens. As a result of reduced colonisation by mycorrhizal fungi, gene-edited

lines may show slightly increased susceptibility to foliar pathogens, while genetically modified lines are likely to show reduced susceptibility to pathogens. There are no adverse environmental effects predicted by changes to these interactions. Other 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,

Due to the potential for minor increases in foliar pathogen susceptibility in the gene-edited lines, plots containing these lines will require additional monitoring and the application of approved fungicide(s) where appropriate. There are no expected adverse environmental effects. Further, the trial will provide an opportunity to investigate any potential changes in the interactions with non-target organisms via observations on disease and pest susceptibility.

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

AMF conization is known to lead to increased drought and salt tolerance. Since the lines in this release will not be subject to drought and salt stress, they will not show any effect on these abiotic stresses. It is not expected that the barley lines for this field trial will give rise to any adverse environmental effects. This trial might provide an opportunity to investigate any potential changes regarding drought and salt tolerance.

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. The gene-edited and gene-modified barley have exhibited a difference in the expression pattern of a number of genes involved in the plant metabolites. None of these genes are known to be toxic or harmful to human health, nor are they known to exert any toxic or allergenic effects.

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 gene-edited plants can be identified by PCR using the already developed gene-specific primers, and subsequent Sanger sequencing assay. The list of the primer sequences can be found in Table 12. Note that the 6 primer pairs provided below are sufficient to facilitate the identification of the 11 gene-edited lines in this trail. The genetically modified plants can be identified through Taqman quantitative PCR assay in which the hygromycin resistance gene (Hyg) and the housekeeping gene Constans (Con) [gene ID: AF490469] get amplified. The list of the primers and probes sequences can be found in Table 13.

Table 12. List of primer sequences to identify the mutations in the 11 gene-edited lines used in this release.

Line description	Forward primers	Reverse primers
symrk-2	cctcctcctcctcgtcttct	acccgcagatcactgtatcc
ccamk-1 & *ccamk-2*	ctcctgccctctaaggctct	agcacagctcgaggatgag
cyclops-2 & *cyclops-3*	tcagtcaatcatcggcagag	ttcctcgaaaggaggaagtg
ram1-1 & *ram1-2*	tcctacgttaacagcagcgagaac	tgcacaaccgcaacctaata
nsp1-1 & *nsp1-4*	ccgattcatccgccaaaccctaaac	gtagaagaggtgctgcacgc
nsp2-2 & *nsp2-4*	caacgtggagacaagaagca	tccttgagccgaaccaatatcac

Table 13. List of primer and probes for Taqman qPCR assay to identify the copy number of transgene.

	Description	Sequences
HvHyg	Forward primer	GGATTTCGGCTCCAACAATG
	Reverse primer	TATTGGGAATCCCCGAACATC
	Probe	Fam-CAGCGGTCATTGACTGGAGCGAGG-Tamra
HvCon	Forward primer	TGCTAACCGTGTGGCATCAC
	Reverse primer	GGTACATAGTGCTGCTGCATCTG
	Probe	VIC-CATGAGCGTGTGCGTGTCTGCG-TAMRA

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.

In each of the five years, the field trials will be conducted on NIAB managed land, in the first year this will be at the Park Farm site at the National Institute of Agricultural Botany (NIAB), Cambridge, UK, grid reference in the region of TL 42949 61941. In subsequent years site grid references in the region of TL 46991 43918 and TL 38108 64920 may also be used. The area for the proposed field trial will be no more than 2100 m^2 and be of similar design to (Figure 10). It will comprise 108 x [1.5 x 4.25 metre] plots planted. Any combination from the 13 lines in the table 11 will be used to make up the total number of trial plots, including the 11 lines gene-edited lines (such as *symrk-2* and *nsp2-2*), and the two overexpression lines (including, OxHvNSP2 (*nsp2-2*) or OxMtNSP2 of barley cv. Golden Promise, and the control barley cv Golden Promise. There will be two treatments; application and no application of phosphorus fertiliser. There will be six replicate plots of each treatment per each barley line combination. The separation between the plots will be 1 metre and there will be a barley pollen barrier of 3 metre surrounding the perimeter of the trial. No cereals or grasses will be cultivated or allowed to grow for a further 20 metres from the outer edge of the pollen barrier. Steel mesh security type fencing will be used to enclose the site to prevent animal access, unauthorised access, and potential vandalism.

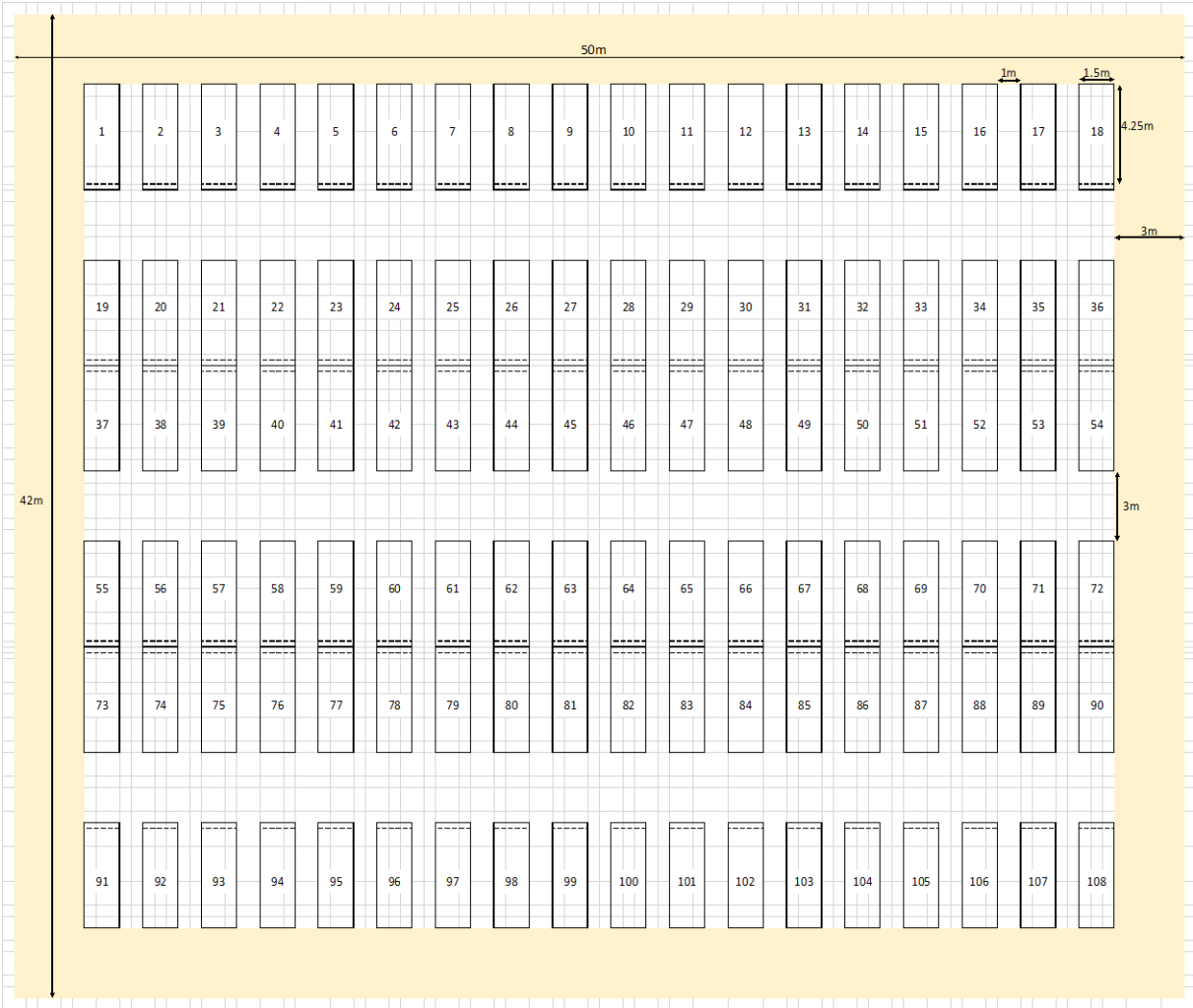


Figure 10. Indicative trial plan layout. 108 plots of 4.25 x 1.5m are indicated, allowing for the testing of 9 genotypes replicated 6 times at 2 fertiliser treatment levels.

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

The release sites are agricultural areas forming part of an experimental farm managed by NIAB's Cambridge Regional centre. Except for the experimental plots themselves, no barley crop will be grown within the accepted separation distance of 20 metres from the release site. The flora and fauna are 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.

Barley cv. Golden Promise is naturally an inbreeding self-pollinating crop with very low rates of cross-pollination with other barley plants. However, the pollination of Golden Promise by its wild relative *Hordeum bulbosum* through breeding in glasshouses can lead to haploid plants [16]. There are no sexually compatible wild barley relatives present on the release site as no barley, other cereals or grasses will be cultivated or allowed to grow within 20 metres 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 1 km of the release sites.

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 arbuscular mycorrhizal fungi (AMF) in barley biomass and yield in the field. Due to the extensive use of phosphorus fertilizers in agricultural practices in the UK, the field soils typically contain high phosphate levels, which prevents crop plants from taking advantage of beneficial associations with natural microbial symbionts such as AMF. The gene-edited lines which will be used in this field trial will reveal the degree to which crop plants such as barley can benefit from naturally occurring mycorrhizal associations in the field. In these gene-edited plants, key genes such as *SYMRK*, *CCamK, Cyclops, RAM1, NSP1,* and *NSP2* are impaired. These genes are involved in the perception and colonisation by AM fungi. Therefore, such mutants are not able to fully take

advantage of AMF colonization. Their impaired yield and growth in the gene-edited lines in comparison to wild-type plants will inform us of the advantages delivered naturally by AMF. Moreover, the overexpression of genetically modified lines (OxHvNSP2 and OxMtNSP2) by their abilities to override phosphate suppression of mycorrhizal colonisation can take advantage of the association with AMF even in the current highly phosphorus-rich soils in the UK. Traits that could be measured from this trial include soil nutrient availability, AMF community composition, leaf chlorophyll content, flowering time, and disease incidence. In short, the field evaluation of transgene-derived traits which alter plant association with AMF will be carried out, with the predominant focus being on enhancement to AMF association in the presence and absence of phosphorus fertilizers.

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

If consent is granted, this year's field trial will start with drilling from mid-February 2022 and harvested by September of the same year. The exact timing of drilling and harvesting of the trial will depend upon weather conditions at the time. The trial will then proceed for four more growing seasons (2023, 2024, 2025, and 2026) for a total of five years.

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

Seeds will be drilled using conventional plot-scale trials equipment and the trial seed drilling equipment will be cleaned after use before removal from the trial site to ensure no seeds are stuck in it.

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 by staff from the NIAB field trials team according to standard agronomic practices for spring barley cultivation. Herbicide treatment and mechanical cultivation will be used to clear the ground prior to drilling. The release will be monitored regularly during all stages of development and harvested at maturity.

Harvest will occur by the end of September depending on weather conditions at the time (if the plants senesce before this then harvesting will be brought forward). Ears (spike/inflorescences) of transgenic and control plants will be harvested using a plot combine harvester. The plot combine will be cleaned at the end of harvesting over a

plastic sheet to ensure no grain is removed from the trial site. Seeds will be stored in appropriate GM seed stores.

The plot will be monitored for volunteer plants immediately following harvest. This will include a shallow light tillage (minimum depth 5 cm) to encourage volunteers in autumn. Non-selective herbicide treatment and/or hand pulling will be used to control any volunteers. The area will be left fallow over winter. In spring a non-selective herbicide applied as required to control any volunteers that are present followed by another shallow light tillage to encourage further germination. The area will be monitored for volunteers during growing seasons (2022- 2026) during which time it will remain fallow. Any volunteers detected in this two-year post-harvest period will be recorded and then destroyed by the application of a non-selective herbicide or by autoclaving any hand pulling plant before ear emergence.

All other materials, including those 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.

96 of the 108 plots will be planted with GM lines, adding up to 612 m^2 (96 × [1.5 x 4.25 m]. Planting density will be approximately 300 seeds per m^2, so the total number of GM plants in the trial each year will be approximately 183,600.

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

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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) Barley is a self-pollinating crop with very low rates of cross-pollination with other barley plants. Crosses between some varieties of barley and some varieties of wheat (*Triticum aestivum L.*) using laboratory techniques resulting in hybrids have been reported [17]. Nevertheless, the outer edge of the trial has a 3-metre-wide strip of non-GM barley to function as a pollen barrier (Figure 10). In addition, no barley, other cereals, or grasses will be cultivated or allowed to grow within 20 metres 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. Various bird-scaring devices 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, fungicides, nitrogen, sulphur and other fertilisers except some of the plots will not receive phosphorus fertiliser. The site will be monitored regularly throughout the trial. Harvest will occur by the end of September depending on weather conditions. The trial will be moved within the field year to year to prevent the plants being affected by pathogens. The plot will be left fallow after harvesting, monitored for the remainder of the year, and sprayed with non-selective herbicides.

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 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, the trial area will remain fallow for the following year to enable monitoring of volunteers and a non-selective herbicide such as glyphosate will be applied as required. Shallow cultivation will be used to encourage volunteers to germinate before treatment with the herbicide. Any volunteers detected in this two-year post-harvest period will be recorded and then destroyed by the application of non-selective herbicide or by autoclaving before ear emergence.

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 barley plants. During the trial, the release site will be visited by trained laboratory personnel who are working on the project at no less than monthly intervals. Visits will usually occur more frequently during growing season, 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 GM 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 will be inspected fortnightly between harvest and November and then once per calendar month for the following 2 years. Any volunteers identified will be immediately destroyed either by application of a non-selective herbicide or by hand pulling plants and digging out the root systems. Hand-pulled volunteers will then be autoclaved. If volunteers are found at the end of the 2-year period, DEFRA recommendations will be followed for the management of the release site.

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 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. Should the release site be subject to vandalism, care will be taken to ensure that all uprooted plant material within and outside of the trial site is identified and destroyed accordingly as described above.

40. Methods and procedures to protect the site.

The NIAB Trials team has experience of previous GM field trials at the site. The release site will be fenced to protect against animal damage. A sign will be posted indicating entry by unauthorised persons is prohibited. The release sites will be securely fenced. A sign will be posted indicating entry by unauthorised persons is prohibited.

Part VIII Information on methodology

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

1. The plasmids for the production of the gene-edited lines and the gene modified lines were constructed using the Golden Gate cloning system [10, 13]. The gene sequences were domesticated and synthesized or PCR amplified, and then cloned into level 0 vector pMS or pMK (GeneArt, Thermo Fisher Scientific). The Golden Gate assembly cloning of the constructs to the level 1 and level 2 vectors were performed following standard protocols [14]. The constructs were transformed into *Agrobacterium tumefaciens* AGL1 in preparation for barley transformation following standard protocols.
2. The T-DNA binary vectors were transformed through the routine transformation protocol for the spring barley Golden Promise [10 & 11], based on *Agrobacterium*-mediated inoculation of immature embryos as described [12].
3. The methods used to create barley CRISPR gene-edited lines are as described [8]. The specific target sequences of guide RNA and the mutation sites in each mutant are listed in Table 1. Characterisation of CRISPR-induced mutations (edits) in the GM plants was performed on DNA that was extracted from leaves using standard protocols.
4. The presence of the *Cas9*, transgene, and gene edition in the plants being grown to provide seed for the field trial was assessed by PCR followed by Sanger sequencing using specific primers. The copy number of the transgenes was identified in each plant generation through TaqMan qPCR assay using primers and probes specific for the Hygromycin resistance gene. The list of the primers can be found in Table 12 and Table 13.
5. For mycorrhization assays, the barley seedlings were inoculated with 10 % crude inoculum (produced on *Tagetes multiflora*) of *R. irregularis* and grown in a cone system at 28°C/20°C,12h light/12h dark cycle with 60% humidity. Plants were watered with ddH_2O only for the first 1-2 weeks, then were nurtured with half-strength Hoagland solution containing 25 µM phosphate twice a week. For nutrient treatments on barley *NSP* overexpression lines, half-strength Hoagland solutions containing a range of phosphate concentrations were given twice a week repeatedly. After 7 or 8 weeks post inoculation, mycorrhizal-colonized roots were harvested and stained by Tryptan blue [18]. Stained roots were mounted on slides, and fungal colonization was quantified at 10 representative random points per root piece.

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