**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.**

**Applicant:**

Project Leader: Scientist with overall responsibility for the application.

**Scientists:**

Project Leader: Scientist who has led the research on the *VIT2* and *NAS* genes and the transgenic high-iron wheat lines.

Field Experimentation Manager: Responsible for the husbandry of the wheat lines.

Research Assistant: Scientist who will be responsible for the day-to-day management and phenotyping of the field experiment.

John Innes Centre
Norwich Research Park
Norwich
NR4 7UH
UK

**2. The title of the project.**

Iron Biofortified Wheat

### Part II Information relating to the parental or recipient plant

**3. The full name of the plant -**

| | |
|---|---|
| **(a) family name** | Poaceae |
| **(b) genus** | *Triticum* |
| **(c) species** | *aestivum* |
| **(d) subspecies** | Not applicable |
| **(e) cultivar/breeding line** | Fielder |
| **(f) common name** | common wheat/bread wheat/ spring 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; and**

The generation time is 20-24 weeks. For Fielder (spring-wheat type), one season is normally from March/April to August in the UK.

**(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 wheat x *Agropyron* spontaneous hybrids. Wheat can also be forced using laboratory techniques to cross to rye, triticale and a limited number of other cereals.

**5. Information concerning the survivability of the plant:**

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

**(b) any specific factors affecting survivability.**

(a and 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’. Fielder is a US spring wheat which has no vernalisation requirement

**6. Information concerning the dissemination of the plant:**

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

**(b) any specific factors affecting dissemination.**

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% (eg. 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 III Information relating to the genetic modification

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

Transgenic wheat plants were produced using the standard protocol by *Agrobacterium*-mediated transformation described in Rey et al 2018. The constructs were introduced to *T. aestivum* cv. Fielder by *Agrobacterium*-mediated inoculation of immature embryos. Whole plants were regenerated and selected from somatic embryos induced in tissue culture.

### 11. The nature and source of the vector used.

Two gene sequences (details below) were cloned into vector pMDC32 (Curtis & Grossniklaus, 2003) which is based on the pCAMBIA backbone, which in turn was derived from the pPZP backbone (Hadjukiewicz et al 1994). These vectors and their derivatives are widely used for plant transformation, enabling the insertion of a so-called T-DNA sequence fragment into a plant genome mediated by *Agrobacterium tumefaciens*.

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

The first gene sequence of interest, the wheat *VACUOLAR IRON TRANSPORTER 2* D genome homoeologue (*TaVIT2-D*; TraesCS5D02G209900 from *Triticum aestivum* L), was cloned into vector pRRes14_RR.301 containing the promoter sequence for the endosperm-specific wheat high-molecular-weight glutenin subunit 1Dx5 (*HMWG*,

Lamacchia et al., 2001). The intended function of the *HMWG-TaVIT2* fragment is to increase expression of the wheat *VIT2* gene in the endosperm of the wheat grain. We showed that this genetic manipulation results in iron accumulation in the vacuoles of endosperm cells around the vascular bundle of the grain (Sheraz et al., 2021) and in >2-fold increased iron in the white flour fraction (Connorton et al., 2017; Balk et al., 2019). Wheat containing the *HMWG-TaVIT2* fragment was released in 2019 and 2021 under consent 19/R52/02.

The second gene sequence of interest, the rice *NICOTIANAMINE SYNTHASE 2* gene, (*OsNAS2*, LOC_Os03g19420 from *Oryza sativa* L.) was placed downstream of the maize (*Zea mays* L.) *UBIQUITIN1* promoter. The *UBI1-OsNAS2* gene fragment was cloned into pMDC32 (described in Beasley et al., 2019). The intended function of this fragment is to increase expression levels of nicotianamine synthase throughout the plant, resulting in elevated levels of nicotianamine and derivatives, natural plant organic compounds that mediate transport of iron, zinc and other divalent metals in the plant phloem. Nicotianamine also improves the bioavailability of iron for uptake in human duodenal cell culture (Eagling et al 2014; Beasley et al., 2019). Wheat containing the *UBI1-NAS2* gene fragment was previously released in Australia from 2015-2021 under DIR128 and DIR152 published by the Australian Government, Department of Health, Office of the Gene Technology Regulator.
https://www.ogtr.gov.au/gmo-dealings/dealings-involving-intentional-release/dir-128
https://www.ogtr.gov.au/gmo-dealings/dealings-involving-intentional-release/dir-152

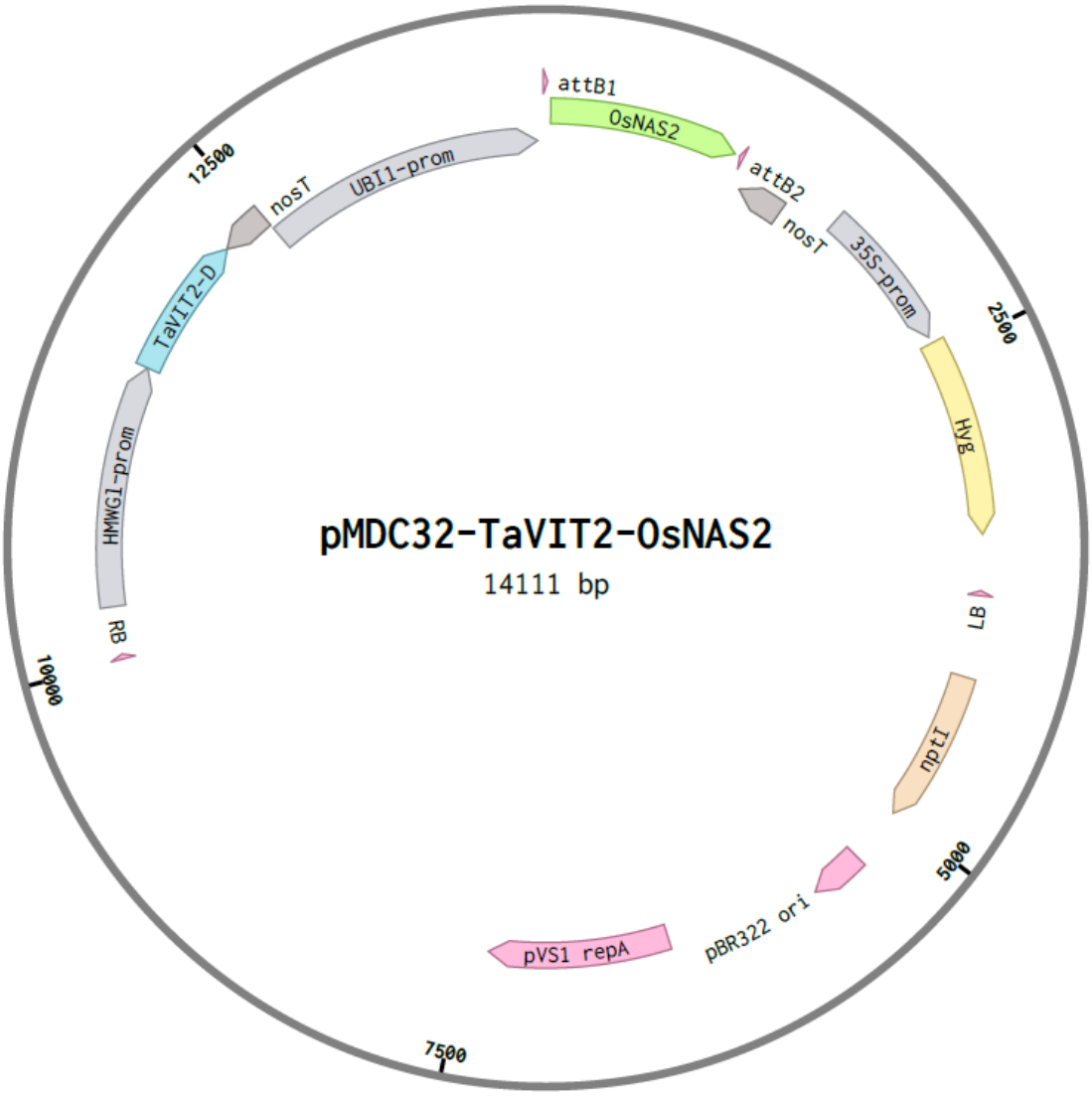
The *HMWG-TaVIT2* fragment was inserted 5' of *UBI1-OsNAS2* into pMDC32 to generate pMDC32-TaVIT2-OsNAS2.

Sizes of all sequence fragments are detailed in the table below.

**pMDC32-TaVIT2-OsNAS2**

| Element | Size (bp) | Donor organism | Description and intended function |
|---|---|---|---|
| *pBR322 ori* | 281 | *Escherichia coli* | Origin of replication for plasmid replication in *E. coli* |
| *pVS1 repA* (plus neighbouring sequence elements) | 1001 | *Pseudomonas aeruginosa* | Replicon for plasmid replication and stability in *Agrobacterium tumefaciens, derived from a Pseudomonas bacterium* |
| *nptI* | 795 | *E. coli* | Bacterial selection gene conferring resistance to kanamycin |
| RB | 26 | *Agrobacterium tumefaciens* | T-DNA right border |

| | | | |
|---|---|---|---|
| LB | 26 | *Agrobacterium tumefaciens* | T-DNA left border |
| 35S-prom | 771 | Cauliflower mosaic virus (CaMV) | Promoter sequence from CaMV |
| *Hyg* | 1026 | *E. coli* and *Ricinus communis* | Plant selectable marker gene encoding hygromycin phosphotransferase gene, including *CAT-1* intron from *Ricinus communis* catalase-1 gene. |
| *UBI1*-prom | 1540 | *Zea mays* | Promoter sequence of the maize *UBIQUITIN1* gene, for high expression of a downstream coding sequence throughout the plant |
| attB1, attB2 | 21 - 25 | - | Sequence elements for Gateway (recombination) cloning |
| *OsNAS2* | 981 | *Oryza sativa* | Coding sequence for *NICOTIANAMINE SYNTHASE* 2 gene, to increase biosynthesis of nicotianamine and its derivates, molecules that bind to iron and zinc to make them more soluble and bioavailable. |
| *nosT* | 258 | *Agrobacterium tumefaciens* | Nopaline synthase terminator |
| *HMWG*-prom | 1256 | *Triticum aestivum* | Promoter sequence for high molecular weight glutenin subunit 1Dx5, for high expression of a downstream coding sequence in the endosperm. |
| *TaVIT2-D* | 738 | *Triticum aestivum* | Coding sequence for *VACUOLAR IRON TRANSPORTER* 2, D genome copy |




**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.**

Increasing the intrinsic nutritional quality of crops, known as biofortification, is viewed as a sustainable approach to alleviate micronutrient deficiencies. In particular, iron deficiency anaemia is a major global health issue, but the iron content of staple crops such as wheat has been difficult to improve using conventional breeding. We have shown that the wheat *VACUOLAR IRON TRANSPORTER 2* gene (*TaVIT2*) functions as an iron transporter (Connorton et al 2017). Overexpression of *TaVIT2* under the control of a wheat endosperm-specific promoter (*HMWG*) increases iron in

white flour fractions by greater than 2-fold, to levels that could replace mandatory chemical fortification (> 16,5 mg/kg), as shown in plants grown under containment (Connorton et al 2017; Balk et al 2019) and in our previously conducted field trials (2019 and 2021; Consent 19/R52/02; manuscript in preparation). The single-gene approach did not affect plant growth as defined by several phenotypic measurements including plant height, tillers per plant, grain number per plant nor grain weight in controlled environment grown plants (Connorton et al 2017). However, we found a 10% decrease in grain size in the 2019 and 2021 field trials.

To further improve the nutritional quality of wheat, we have generated a new line containing the *TaVIT2* cassette and the *OsNAS2* cassette developed by Alex Johnson at the University of Melbourne, Australia (Beasley et al 2019). Johnson and colleagues showed that overexpression of the rice (*Oryza sativa*, *Os*) *NICOTIANAMINE SYNTHASE* (*NAS*) gene under the control of the maize *UBIQUITIN1* promoter (*UBI1*; for ubiquitous expression in the plant) resulted in ~10% higher iron and ~20% higher zinc in whole grains, but most importantly, they demonstrated a 10-fold increase in nicotianamine correlated with improved bioavailability of iron (Beasley et al 2019).

Having selected and analysed five independent lines with stable insertion of the *HMWG-TaVIT2* and *UBI1-OsNAS2* construct (*TaVIT2* + *OsNAS*), we find, in controlled environmental conditions, that the effect of the two genes is additive, resulting in >2-fold iron in the white flour fraction, increased zinc in white flour and bran fractions, and up to 10-fold more nicotianamine. Bioavailability of the minerals has not been tested, because of the limited amount of material grown in the greenhouse. This is why field trials will be important, to obtain enough grain for milling and quality determinations, and also to test the growth of the plants in the field.

The pMDC32-TaVIT2-OsNAS2 plasmid also contains the *nptI* kanamycin resistance gene for selection of transformed bacteria, and the *Hyg* gene for selection of transformed plants (under control of the cauliflower mosaic virus 35S promoter). Only the *Hyg* gene is present in stably transformed lines, the *nptI* gene is generally not inserted into the plant genome and subsequently lost.

This application seeks authority to investigate the effects on enhancing micronutrient accumulation in the grain by over-expressing the wheat *TaVIT2* gene in the endosperm and the rice *OsNAS2* gene in wheat plants in the field.

**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.**

We propose to grow three separate transgenic lines in the field trial, each transformed with the same gene construct, and one control line (null segregant). The grain is the so called $T_3$ generation of the transformation events. Line B4 contains 2 copies of the TaVIT2-OsNAS2 T-DNA per haploid genome, line B12 contains 6 copies and line B14 contains 3 copies, as determined by quantitative (Taqman) PCR for the *Hyg* gene performed on genomic DNA by iDNA Genetics (Norwich, UK). Line A1 does not contain any copies of the construct, but was generated during the transformation procedure, and has thus undergone the same treatments to serve as a suitable control line. Plants were transformed using *A. tumefaciens*; therefore, all transformation events will result in a nuclear location for the transgenes.

We have not analysed the position or the structure of the insertion nor sequenced the flanking genomic DNA. Apart from the expected phenotype of increased iron content in the endosperm (checked by Perls' staining and confirmed by ICP-OES analysis), plants from the three transgenic lines are indistinguishable from untransformed controls, when grown in glasshouses or in controlled environment rooms. No other changes to the plant morphology or development are apparent, indicating that no genes essential for growth are potentially disrupted by the inserted T-DNA.

**15. The following information on the expression of the insert**

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

Segregation analysis using PCR of genomic DNA indicates that in all lines, all the gene insertions are carried in the chromosomal DNA and are stably inherited. The three lines are homozygous.

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

The *TaVIT2* and the hygromycin resistance genes are under the transcriptional control of the wheat *High Molecular Weight Glutenin-D1* (*HMWG*) gene promoter (Lamacchia et al 2001) and the cauliflower mosaic virus promoter 35S (+ intron),

respectively, as in our previous release 19/R52/02. The *OsNAS2* gene is under the transcriptional control of the maize *UBIQUITIN1* gene promoter (Beasley et al 2019).

The expression of (transgenic) *TaVIT2* and *OsNAS2* has been verified by RT-qPCR, and is as expected, namely *TaVIT2* transcripts are 5 – 15-fold increased in the grain compared to endogenous *TaVIT2*, and *OsNAS2* transcripts are highly abundant in grain and leaf tissues (manuscript in preparation). The wheat *HMWG* promoter is specific to the endosperm of developing wheat grains and expression is not observed in any other vegetative tissue during the life cycle of the plant.

## 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,

In our previous release (19/R52/02; 2019 and 2021) we did not see an effect of the single *TaVIT2* gene on plant height, tillers per plant, or grain number per plant. We did find a 10% decrease in grain size in the 2019 field trial. For the *OsNAS2* transgenic lines grown in Australia (2015-2017; 3 locations), no effects were observed for grain size or plant yield, although a small but consistent reduction in plant height was observed.

Based on our controlled growth environment trials of the *TaVIT2-OsNAS2* plants, pollination and seed-set appear to be similar to non-transgenic control wheat plants. No difference in fertility, seed size or germination was observed from these plants. Vegetative performance of the transgenic plants was unaltered.

We would expect dissemination of pollen to be the same as for non-transgenic wheat plants given the results from our controlled growth environments experiments, the very localised expression of the *HMWG* promoter and previous release 19/R52/02 (for *VIT2*) and the experience of the *OsNAS2* plants across 9 trials (3 years x 3 locations) in Australia. We do not expect any changes in survivability of these plants in unmanaged systems.

The probability of seeds escaping from the trial site or transfer of inserted genetic material to sexually-compatible species outside the trial area is estimated as very low. Commercial wheat cultivars do not establish easily or thrive in uncultivated environments and are naturally self-pollinating with out-crossing being a rare event. Wheat seeds are relatively large and not normally dispersed by wind. Management procedures to minimise the spread of seeds or pollen (e.g 2 m pollen barrier surrounding trial) will further reduce the probability of these events occurring. Appropriate physical barriers (fenced growing area and full height netted framework over experimental planting) will be employed to prevent access by mammals and

birds. There will be no cereals grown for 20 metres from the boundary of the experimental plots and no sexually-compatible wild relatives of wheat exist in the vicinity.

It is highly unlikely that intended or unintended effects of the genetic modification of increased endosperm iron content will result in major changes in invasiveness or persistence. The genes introduced into the plants proposed for release do not confer characteristics that would increase the competitiveness of plants in unmanaged ecosystems. Plants remain sensitive to all herbicides such as glyphosate or glufosinate. The introduced genes are thus not anticipated to confer any intrinsic advantage compared to conventional wheat cultivars with respect to persistence in agricultural habitats or invasiveness in natural habitats and no emergent hazard is predicted.

**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 non-sexual, horizontal gene transfer to other species is extremely low. In the event of horizontal gene transfer to bacteria, neither the trait gene nor the selectable marker genes would be expected to confer a selective advantage in the field environment under consideration. The plasmid backbone sequences, *nptI* gene, origins of replication, border sequences etc. come originally from *E coli* and *Agrobacterium tumefaciens*, two common gut and soil bacteria respectively and these sequences are already widespread in the soil metagenome. Although this makes potential homologous recombination events more likely, we estimate the likelihood of horizontal gene transfer as low and the consequences, were it to occur, negligible. The area proposed to be planted with GMOs is small and temporary lasting between 5 to 6 months during the three years (2022-2024).

Although both the above-ground plant material and roots will be cleared from the site, the *nptI* gene contained in the remaining plant root DNA will decompose into the soil. The transgene is fully integrated into the plant DNA and the copy number is low thus the nptI gene represents a very small proportion (much less than one millionth) of the total DNA in any one cell of the transformed wheat plants. This excess of competing DNA will significantly dilute the rate of any *nptI* natural bacterial transformation. In addition, enzymatic degradation of free plant DNA in the soil and the low level of spontaneous bacterial competence to take up free DNA will significantly reduce the incidence of natural transformation. Although the transfer of functional gene units from plants to soil bacteria is accepted to be extremely low under natural conditions (Schluëter et al 1995, Nielsen et al 1997, EFSA, 2009), it cannot be completely discounted that some bacteria may successfully take up the *nptI* gene. However, there will be no antibiotics applied to the soil to provide

additional selection pressure for the gene to persist in the environment. The source of the *nptI* gene is the gut bacterium *E. coli* carrying a plasmid containing the transposable element (Tn 903). R plasmids possessing resistance to aminoglycoside antibiotics are already naturally found in the soil and other environments. The *nptI* gene encodes the enzyme aminoglycoside 3'-phosphotransferase which confers resistance to kanamycin and related aminoglycoside antibiotics. Although these antibiotics still have some clinical applications, alternatives are readily available. Taken together and bearing in mind the limited scope of this trial, the risk of generating of any additional antibiotic resistance within the soil microbial community or risks to human health or the environment if this were to occur as a result of the proposed trial is considered to be extremely low.

**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 no-target organisms resulting from the genetic modification and the adverse environmental effects arising,**

There are no known or predicted interactions associated with this trait and non-target organisms.

**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,**

There are no known or predicted changes in agricultural practices and management resulting from the genetic modification.

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

There are no known or predicted interactions associated with this trait and abiotic stress.

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

The VIT2 protein occurs naturally in wheat and across many other plants and fungi. In this transgenic line we have expressed the native wheat *VIT2* gene ectopically in the wheat endosperm (using a native wheat promoter). There appears to be no published toxicity or allergenicity data for VIT2 but at the levels expected to be generated by these plants and because they will not enter the general food or feed chains, we consider the potential toxic or harmful effects to be negligible. No negative impacts were recorded in release 19/R52/02.

The NAS2 protein also occurs naturally in rice and across many other plants. Similar to VIT2, there appears to be no published toxicity or allergenicity data for this protein. However, at the levels expected to be generated by these plants and because they will not enter the general food or feed chains, we consider the potential toxic or harmful effects to be negligible. No adverse effects on human health and safety (or the environment) were recorded during the multi-site multi-year trial in Australia (https://www.ogtr.gov.au/what-weve-approved/dealings-involving-intentional-release).

The plasmid used possesses two antibiotic resistance genes (*nptI* and *Hyg*) and we have assumed that these are integrated into the plant genomic DNA along with the genes of interest. These elements may increase the rates of horizontal gene transfer and establishment in soil bacteria because they provide a theoretical mechanism for homologous recombination and selection (if appropriate antibiotics are present). The *neomycin phosphotransferase I* (*nptI*) gene is under the control of a bacterial promoter to facilitate the maintenance and replication of plasmid vectors containing the genes of interest in dividing bacterial cells before they are used to transform plant cells. Their expression in plant cells is very unlikely and the gene is already widely present in the environment. The *hygromycin phosphotransferase* (*Hyg*) gene is under the control of a constitutive 35S promoter to facilitate the selection of transgenic plants after transformation. According to EFSA (EFSA 2009) genes conferring resistance to hygromycin are included in the first antibiotic resistance marker genes (ARMG) group. They state that, “with regard to safety there is no rationale for inhibiting or restricting the use of genes in this category, either for field experimentation or for the purpose of placing on the market.” It is worth mentioning that *nptII* (which is closely related to *npt1*) is also classified alongside the *Hyg* gene in the EFSA guidelines.

**23. Conclusions on the specific areas of risk.**

In summary, these sequences are not known to be pathogenic or allergenic to humans, and none of the genes under investigation, or the selectable marker genes, are expected to result in the synthesis of products that are harmful to humans, other organisms or the environment. 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.**

PCR primers for specific detection of the introduced T-DNAs are available and details are given in PART VIII (Section 41).

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

Wheat lines with either the *HMWG-TaVIT2* or the *UBI1-OsNAS2* fragments, combined in the new lines, have previously been released. We previously released wheat lines carrying the *HMWG-TaVIT2* fragment under release 19/R52/02. These plants were grown in two years of field trials (2019 and 2021) with no risks to human health or the environment identified by the GMI Inspectorate (https://www.gov.uk/guidance/gm-inspectorate-deliberate-release-inspection-programme). Wheat with the *UBI1-OsNAS2* sequence was previously released in field trials in Australia from 2015 to 2021. Again, there were no reports of adverse effects on human health and safety or the environment resulting from their release (https://www.ogtr.gov.au/what-weve-approved/dealings-involving-intentional-release).

**Part V Information relating to the site of release**

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

In each of the three years, the transgenic plants will be released on an area of arable land no larger than 50 metres squared located 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 for GM experiments (total area of 17,600 $m^2$). The transgenic plots will be surrounded by a 2 m pollen barrier which will increase the total area to up to 250 $m^2$ (with ~200 $m^2$ of non-GM pollen barrier and gaps and up to 50 $m^2$ of GM plants). In accordance with wheat planting practice, the 250 $m^2$ site will rotate within the release site each year of the trial, therefore a total of 750 $m^2$ will be used for the release across the three years, with a total of < 150 $m^2$ corresponding to GM plants across the three years and the other 600 $m^2$ corresponding to the pollen barrier and gaps between plots. In each year, no cereals or grass species will be cultivated or

allowed to grow for 20 m from the outer edge of the release area (i.e. the pollen barrier).

**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 peas. Except for the experimental plots themselves, no wheat crop 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 VI Information relating to the release

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

The purpose is to investigate the effects of the ectopic over-expression of the wheat *TaVIT2* gene in the endosperm of wheat grains combined with overexpression of the rice *OsNAS2* gene in the whole wheat plant and determine the effect on micronutrient accumulation and agronomic performance in the field. The grain and flours milled from the grain will be subjected to laboratory investigations into the bioavailability of iron and zinc.

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

If consent is granted, the first field trial will start in Spring 2022. The plants will be transplanted in March/April and harvested in August/September. The exact timing of harvesting of the trial will depend upon weather conditions at the time. The trial will then proceed for two more growing seasons (2023 and 2024).

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

Seedlings of transgenic lines will be grown under controlled glasshouse conditions and will be transplanted in the field by hand.

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

The ground will be prepared by members of the John Innes Centre (JIC) Field Experimentation team who carry out field work on the Church Farm site, according to normal agricultural practices for spring wheat cultivation. Ground preparations will consist of existing plants being sprayed with herbicide to clear the ground. Compost

will be applied if necessary and the ground will be prepared for planting using shallow mechanical cultivation. All machinery (including wheels and tyres) used on the trial site will be cleaned thoroughly before leaving the trial site.

Harvest will occur August/September depending on weather conditions at the time (if the plants senesce prior to this then harvesting will be brought forward). Ears (spike/inflorescences) of transgenic and control plants will be hand harvested, conditioned, and manually threshed in a separate designated area, with seeds being stored in appropriate GM seed stores. All personnel entering the trial site will take appropriate steps to eliminate transfer of GMOs via clothing from the trial site.

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. If volunteers are present we will apply systemic herbicide (glyphosate) to ensure their destruction. The area will be left fallow over winter. In spring another shallow light tillage will be performed and glyphosate applied in case any volunteers are present. The area will be monitored for volunteers in the growing season and the following two seasons during which time it will remain uncropped/fallow. Any volunteers detected in this two-year post-harvest period will be recorded and then destroyed by application of glyphosate or by autoclaving before ear emergence.

These methods are all in alignment with previous release 19/R52/02.

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

We will hand transplant lines in the field and sow them as 10 plants per 1 m row. We normally separate rows by ~16 cm (6 rows per meter), therefore the plant density will be ~60 plants/$m^2$. We propose to include three transgenic events which in total will sum to fewer than 2,000 GM wheat plants being grown in each year of the trial.

## 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

**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**: To avoid dispersal of seed while sowing, we will hand-transplant seedling plants grown in controlled environment conditions. Ears of all transgenic lines and controls for all replications will be hand-harvested, conditioned and threshed for phenotypic assessment and research purposes. Any remaining grain will be disposed by autoclaving and disposing in agreement with JIC standard operating procedures for 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. Full height framework and netting will protect the planting from birds and mammals throughout the growing season (from transplant to harvest).

A wheat pollen barrier of at least 2 metres width surrounding the GMOs, at the same or greater density as the GMOs and using a variety or varieties that flower(s) at the same time as the GMOs, will be transplanted on the same day as the GMOs or sown to coincide with the flowering of the GMOs, within the perimeter of the trial site.

Prior to planting, seedlings will be transported from JIC controlled environment rooms to the release site and the plants will not be mixed with either other plants or with equipment used for working on other agricultural land. 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.

These measures have all been put in place during release 19/R52/02.

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

Harvest will occur August/September depending on weather conditions at the time (if the plants senesce prior to this then harvesting will be brought forward). Ears (spike/inflorescences) of transgenic and control plants will be hand harvested, conditioned, and manually threshed in a separate designated area with seeds being 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. The area will be left fallow over winter and in spring another shallow light tillage will be performed and glyphosate applied in case any volunteers are present. The area will be monitored for volunteers in the two growing season following the trial during which time it will remain uncropped/fallow. Any volunteers detected in this two-year post-harvest period will be recorded and then destroyed by application of glyphosate or by autoclaving before ear emergence.

All machinery (including wheels and tyres) used on the trial site will be cleaned thoroughly before leaving the trial site.

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

Ears (spike/inflorescences) of transgenic and control plants will be hand harvested, conditioned, and manually threshed in a separate designated area. Seeds will be stored in appropriate GM seed stores.

GM plants and controls will be pulled from the ground and all straw and as many roots as possible will be placed in sealed, labelled bags or containers, disposed by autoclaving and disposing in agreement with JIC standard operating procedures for transgenic material.

Harvesting of the pollen barrier will be carried out by hand. As for 19/R52/02, we will harvest the pollen barrier (plants and developing grains) after the GM plants have finished flowering, and before harvesting the experimental plots. This will allow the pollen barrier to fulfil its function, and at the same time minimise the chance of mature seeds from the pollen barrier falling during harvest. Aerial material will be

placed in sealed, labelled bags or containers, disposed by autoclaving and disposing in agreement 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 laboratory personnel who are working on the project once every 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. Keys will be held at the John Innes Centre (JIC) reception and at the Dorothea de Winton Field Station at Church Farm. We will restrict human access to the trial site to only those personnel who have been informed of the limitations and conditions of the consent. We will include additional measures for birds and rabbits by enclosing the planting in framework and netting throughout the growing season. The site will also include a beam perimeter security alarm which will connect to the JIC reception which is manned throughout the day by JIC reception staff and by security guards out of normal working hours. GM Inspectorate will have access to the trial site on request.

## Part VIII Information on methodology

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

From Connorton et al 2017, “The *TaVIT2* gene was amplified by PCR and, using restriction cloning, inserted into vector pRRes14_RR.301 containing the promoter sequence comprising nucleotides -1,187 to -3 with respect to the ATG start codon of the *HMW GLU-1D-1* gene (*HMWG*), which encodes the high-molecular-weight glutenin subunit 1Dx5 (Lamacchia et al 2001).” Next, using InFusion cloning, the *HMWG-TaVIT2* fragment was inserted in the pMDC32 plasmid, which already contained *UBI1-OsNAS2* and the *Hyg* resistance marker. The cloning of the pMDC32-OsNAS2 plasmid is described in Beasley et al 2019, “The full-length coding sequence of *OsNAS2* (LOC_Os03g19420) was PCR amplified from rice (*Oryza sativa* L.) cv. Nipponbare genomic DNA. [This was inserted into] the pMDC32 vector (Curtis and Grossniklaus, 2003) containing the hygromycin phosphotransferase (*Hyg*) plant-selectable marker gene, [by Gateway recombination cloning]. *OsNAS2* was placed under transcriptional control of the maize (*Zea mays* L.) *UBIQUITIN 1* promoter”. The construct was checked by DNA sequencing.

Transformation into wheat (cv Fielder) was performed by the BRACT platform at the John Innes Centre using *Agrobacterium tumefaciens*-mediated techniques as described previously (Wu et al 2003; Harwood et al 2009). Transgene insertion and copy number in $T_0$ plants were assessed by iDNA Genetics using quantitative PCR

with a Taqman probe. For the $T_1$ generation, the presence of the hygromycin resistance gene was analyzed by PCR with primers Hyg-F (ATGAAAAAGCCTGAACTCACC) and Hyg-R (CTATTCCTTTGCCCTCGGA).

In-depth analysis by the Balk laboratory of transformed plants, of the $T_1$, $T_2$ and $T_3$ generations grown in controlled environment rooms, showed that the intended trait changes were realised, namely increased expression of the *TaVIT2* and *OsNAS2* genes was shown by Reverse Transcription-qPCR; increased nicotianamine levels were shown by metabolite analysis (LC-MS); and increased iron and zinc in white flour and bran fractions of the grain was shown by element analysis (ICP-OES). The draft manuscript describing these data is being finalised (current version available upon request), and is anticipated to be posted on the open science server *bioRxiv* by January 2022, with subsequent peer-review and publication.

## References


Balk J, Connorton JM, Wan Y, Lovegrove A, Moore KL, Uauy C, Sharp PA and Shewry PR (2019). Improving wheat as a source of iron and zinc for global nutrition. Nutr. Bull. 44:53-59.

Beasley JT, Bonneau JP, Sánchez-Palacios JT, Moreno-Moyano LT, Callahan DL, Tako E, Glahn RP, Lombi E, Johnson AAT (2019) Metabolic engineering of bread wheat improves grain iron concentration and bioavailability. Plant Biotechnology Journal. 17:1514-1526.

Connorton JM, Jones ER, Rodriguez-Ramiro I, Fairweather-Tait S, Uauy C, Balk J (2017) Vacuolar Iron Transporter TaVIT2 transports Fe and Mn and is effective for biofortification. Plant Physiology 174:2434-2444.

Curtis, MD and Grossniklaus, U (2003) A Gateway cloning vector set for high throughput functional analysis of genes in planta. Plant Physiol. 133, 462–469.

Eagling T, Wawer AA, Shewry PR, Zhao FJ, Fairweather-Tait SJ (2014) Iron bioavailability in two commercial cultivars of wheat: comparison between wholegrain and white flour and the effects of nicotianamine and 2'-deoxymugineic acid on iron uptake into Caco-2 cells. J Agric Food Chem. 62:10320-5.

EFSA (2009) Use of Antibiotic Resistance Genes as Marker Genes in Genetically Modified Plants. The EFSA Journal 1034:66-82.

Griffin WB (1987) Out crossing in New Zealand wheats measured by occurrence of purple grain. N.Z Journal of Agricultural Research 30:287-290.

Hajdukiewicz P, Svab Z, Maliga P (1994) The small, versatile pPZP family of Agrobacterium binary vectors for plant transformation. Plant Mol Biol. 25:989-94. doi: 10.1007/BF00014672.

Harwood WA, Bartlett JG, Alves SC, Perry M, Smedley MA, Leyland N, Snape JW (2009) Barley transformation using Agrobacterium-mediated techniques. In HD Jones, PR Shewry, eds, Transgenic Wheat, Barley and Oats. Humana Press, Totowa, NJ, pp 137–147

Hellens RP, Edwards EA, Leyland NR, Bean S, Mullineaux PM (2000) pGreen: a versatile and flexible binary Ti vector for *Agrobacterium*-mediated plant transformation. Plant Molecular Biology 42:819–832.

Hucl, P (1996) Out-crossing rates for 10 Canadian spring wheat cultivars. Canadian Journal of Plant Science. 76:423–427.

Lamacchia C, Shewry PR, Di Fonzo N, Forsyth JL, Harris N, Lazzeri PA, Napier JA, Halford NG, Barcelo P (2001) Endosperm-specific activity of a storage protein gene promoter in transgenic wheat seed. Journal of Experimental Botany 52:243–250.

Martin TJ (1990) Out crossing in twelve hard red winter wheat cultivars. Crop Science 30:59-62.

Rey MD, Martin AH, Smedley M, Hayta S, Harwood W, Shaw P, Moore G (2018) Magnesium Increases Homoeologous Crossover Frequency During Meiosis in *ZIP4* (*Ph1* Gene) Mutant Wheat-Wild Relative Hybrids. Frontiers in Plant Science. 9:509.

Sheraz S, Wan Y, Venter E, Verma SK, Xiong Q, Waites J, Connorton JM, Shewry PR, Moore KL and Balk J (2021) Subcellular dynamics studies of iron reveal how tissue-specific distribution patterns are established in developing wheat grains. New Phytol 231: 1644-1657. doi: 10.1111/nph.17440

Wu H, Sparks C, Amoah B, Jones HD (2003) Factors influencing successful Agrobacterium-mediated genetic transformation of wheat. Plant Cell Reports 21:659–668.