Part B: Information about the release application to be included on the public register

B1 The name and address of the applicant

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B2 A general description of the genetically modified organisms in relation to which the application is being made

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 plasmid for the proposed release (pMDC32-TaVIT2-OsNAS2) 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.

B3 The location at which the genetically modified organisms are proposed to be released

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.

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. For each year of the field trial, we estimate that the release will not exceed 2,000 transgenic plants.

B4 The purpose for which the genetically modified organisms are proposed to be released (including any future use to which they are intended to be put).

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. Specific questions to be examined are:

1) Does the over-expression of *TaVIT2* and *OsNAS2* lead to increased iron and zinc accumulation in specific parts of the grain in field-grown plants (e.g., as seen in greenhouse/controlled environment conditions)?

2) Does the over-expression of *OsNAS2* lead to increased nicotianamine levels in wholemeal and white flours (e.g., as seen in greenhouse/controlled environment conditions)?

3) Is there any change to the content of other micronutrients in the grain when the transgenic lines are grown in the field (e.g., phosphorus, manganese)?

4) Is there any effect of over-expressing *TaVIT2* and *OsNAS2* on basic agronomic performance (e.g., phenology, yield components)

5) Is there any difference in agronomic performance between the different transgenic lines?

6) Are the effects consistent across years?

B5 The intended dates of the release.

If consent is granted, we intend to conduct the first trial starting in Spring/Summer 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).

B6 The environmental risk assessment.

Summary
Bearing in mind the limited scope of the trial and that the GM wheat will not be put into the human food chain or fed to livestock, the overall risk of harm to human health or the environmental arising from this trial is assessed as very low.

Environmental risks

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 nor 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 will further reduce the probability of these events occurring (e.g 2 m pollen barrier surrounding trial site). 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 will result in major changes in invasiveness or persistence. The two genes introduced into the plants proposed for release do not confer characteristics that would increase their competitiveness in unmanaged ecosystems. Previous field trials with transgenic lines overexpressing either *TaVIT2* or *OsNAS2* individually did not identify increase in seed size, plant growth, or vigour. Apart from the expected phenotype, which is increased iron and zinc content in the grain endosperm, plants from the three proposed events (combining both *TaVIT2* and *OsNAS2* genes) 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. 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.

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 *Escherichia 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 (total area ≤50 m^2) and temporary lasting between 5 to 6 months during the three years (2022-2024).

Although the above-ground plant material will be cleared from the site and as many roots of the GM plants will be pulled as possible, 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.

Human health risks

The gene donor organisms are cereals grown as staple crops. The *HMWG* promoter and *TaVIT2* coding sequences are from wheat (*Triticum aestivum*) cultivars. The *UBIQUTIN1* promoter seqeunce is from maize (*Zea mays*) and the NAS2 gene is from rice (*Oryza sativa*). Thus, people and other organisms have a long history of exposure to the gene products. 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. Any unknown hazards arising from the expression and ingestion of foreign proteins will not occur since the wheat plants and grains will not be consumed by humans nor will enter the animal food chain.

Apart from the *TaVIT2* and *OsNAS2* genes, the vector also includes the *nptI* and *Hyg* genes. The source organism for the gene encoding the hygromycin phosphotransferase (*Hyg*) enzyme, *Eschericia coli*, is present in the large intestine of healthy humans and there have been no reports of its adverse effects on humans, animals or plants. The product of the *Hyg* gene, hygromycin phosphotransferase, has been evaluated on numerous occasions by EFSA and found to raise no safety concerns. 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." The *neomycin phosphotransferase I* (*nptI*) gene is under the control of a bacterial promoter and is used for bacterial selection only (i.e. before they are used to transform plant cells). The source organism for the gene encoding this enzyme (*E. coli*) is present in the large intestine of healthy humans and any NPTI ingested is expected to be broken down by digestive enzymes in the stomach and small intestine. The expression of NPTI in plant cells is very unlikely and the gene is already widely present in the environment.

B7 The methods and plans for monitoring the genetically modified organisms and for responding to an emergency.

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.

Emergency procedures: 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.