Department for Environment, Food and Rural Affairs

Application for consent to release a GMO – Higher plants

Part A1: Information require under Schedule 1 of the Genetically Modified Organisms (Deliberate Release) Regulations 2002

Part 1: 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 at the John Innes Centre since 2005 and with 14 years of experience working on Brassica developmental genetics. The group is developing genetic resources for Brassica research and is using both molecular genetics and functional genomics to study yield traits.

Scientists:

Transformation / Genome Editing Group Leader, John Innes Centre with 25 years' experience of overseeing the development of transformation and genome editing systems for the major UK crops (wheat, barley, oilseed rape and Brassica oleracea).

Field Experimentation Manager, John Innes Centre with with 29 years' experience in cereal breeding, agriculture and field experimentation.

Senior Researcher, Crop Transformation Group, John Innes Centre with 24 years' experience in Brassica transformation.

John Innes Centre

Norwich Research Park

Colney Lane

Norwich

NR4 7UH

UK

2. The title of the project.

Genetic regulation of sulphur metabolism in *Brassica oleracea*

Part II: Information relating to the parental or recipient plant

3. The full name of the plant -

(a) family name Brassicaceae

(b) genus Brassica

(c) species *B. oleracea*

(d) subspecies *alboglabra* and *italica*

(e) cultivar/breeding line AG DH1012, derivatives, commercial broccoli (*ssp italica*)

AG DH1012 is a doubled haploid genotype from the *Brassica oleracea* ssp *alboglabra* (A12DHd) and *Brassica oleracea* ssp *italica* (Green Duke GDDH33) mapping population (Bohuon et al., 1996; Bohuon, 1995)

(f) common name Chinese Kale/Broccoli

4. Information concerning -

(a) **the reproduction of the plant:**

(i) **the mode or modes of reproduction,**

Reproduction is sexual leading to formation of seeds. *Brassica oleracea* is highly self-compatible though not exclusively self-pollinating with the potential for pollination to be carried out by insects.

(ii) any specific factors affecting reproduction

Flowering, pollination and seed set are dependent on temperature, weather conditions, agronomic practice and pressure applied by pests and disease.

(iii) generation time; and

56 – 70 days (8 -10 weeks)

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

(c) “Within the Brassica genus, successful hybridisation has been reported between *B. oleracea* and several members of the *B. oleracea* cytodeme: *B. bourgeaui, B. cretica, B. incana, B. insularis, B. macrocarpa, B. montana, B. rupestris,* and *B. villosa*. Outside of the cytodeme *B. oleracea* is also capable of successful hybridisation with the crop species*, B. juncea, B. napus* and *B. rapa* and with *B. maurorum*. Successful intergeneric hybridisation has been reported for crosses between B. oleracea and the following species: *Eruca vesicaria, Erucastrum abyssinicum, Hirschfeldia incana, Moricandia arvensis, Raphanus sativus* and *S. arvensis.*” (FitzJohn et al., 2007) These plant species can be found throughout Europe.

5. Information concerning the survivability of the plant:

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

Brassica oleracea is a biennial or perennial herb and survives only via seed production, as it is unable to clonally propagate. Under agricultural practice, in this instance the broccoli inflorescence is removed during an early bud stage, prior to flowering and pollen release. It is possible some mature seeds of flowers produced from axillary branches may remain and be lost into the soil. If not managed, these seeds could potentially over-winter in the soil and germinate the following spring as ‘volunteers’. However, little empirical data are available regarding the proliferation of *Brassica oleracea* plants following the harvest of vegetative material within the UK commercial farming.

(b) any specific factors affecting survivability.

None of note

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 the wind or by insect pollinators, however in this trial the inflorescence would be harvested prior to pollen emergence, and the plants destroyed which would prevent pollination and seed dispersal. Even so, up to 10 plants will be allowed to flower for seed production. These will be contained in pollen-proof bags to prevent pollen and seed dispersal.

(b) any specific factors affecting dissemination.

Brassica oleracea is reported to be as attractive to bees as other species such as flax and canola. However, the flowers of *Brassica oleracea* are smaller than canola or flax, so may be less apparent. Dispersal of seed prior to harvest by wind is unlikely, but possible by wildlife. However, the majority of plants in this trial will be removed before full emergence of flowers, with the exception of some contained in pollen-proof bags which will be applied and tied prior to floral opening.

7. The geographical distribution of the plant.

Brassica oleracea is native to the Mediterranean region and southwestern Europe, as far north as southern England. It is found growing wild on seaside cliffs. Its native locations include France (France (mainland), Germany, Spain (mainland), The Canary Islands and the United Kingdom. However, its commercial use as a crop has led to its introduction across Africa, Asia and both North and South America, including: USA, Mexico, Guatemala, Ecuador, Morocco, Algeria, Tunisia, Libya, DR Congo, Ethiopia, Kenya, Tanzania, Saudi Arabia, India, China, Koreas.

8. Where the application relates to a plant species which is not normally grown in the United Kingdom, 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.

Brassica oleracea is known to have a range of pests and fungal pathogens. The main insect pests in the UK are likely to be Crucifer Flea Beetle (*Phyllotreta cruciferae*) andStriped flea beetle (*Phyllotreta striolata*). Other potential pests include cabbage root fly (*Delia radicum*) and the diamondback moth (*Plutella xylostella*). Fungal pathogens may include the following: *Plasmodiophora brassicae*, *Leptosphaeria maculans*, *Fusarium oxysporum* f. sp. *Conglutinans*, *Albugo candida*, *Botrytis cinere*, *Fusarium* spp, *Mycosphaerella brassicicola*, *Peronospora parasitica*, *Pythium debaryanu*, *Rhizoctonia solani*, *Sclerotinia sclerotiorum*, *Ustilago* spp., *Verticillium longisporum*.

Part III: Information relating to the genetic modification

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

The CRISPR *Brassica oleracea* lines were produced via *Agrobacterium*-mediated transformation using 4-day old cotyledonary explants, as described in https://www.jic.ac.uk/app/uploads/2018/11/Brassica-Transformation.pdf. This is an updated version of the method published in (Sparrow et al., 2006a).

Details of CRISPR protocol used for *Brassica oleracea* gene editing published as (Lawrenson et al., 2015)

11. The nature and source of the vector used.

The vector used is an R2K plasmid derived from E. coli. The backbone of the plasmid is pAGM8031 which comes from the original moclo kit described by (Engler et al., 2014)

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

Element	Size	Donor Organism	Description and Intended Function
RB	128	*Agrobacterium tumefaciens*	T-DNA Right border
LB	151	*Agrobacterium tumefaciens*	T-DNA Left border
CDS			
spec	1008	*E. coli*	Antibiotic resistance gene for selection on media
NPTII	793	*E. coli*	neomycin phosphotransferase II enzyme which allows transformed plants to metabolize neomycin and kanamycin antibiotics during selection
Cas9	4140	*Streptococcus pyogenes*	RNA-guided DNA endonuclease
Guides 1-4	20	*Brassica oleracea*	Guide sequences which target Cas9 to gene of interest
Promoters			
2x CaMV 35S promoter	753	*Cauliflower mosaic virus*	35S Promoter
U6 26 Promoter	206	*Arabidopsis thaliana*	Used to drive the expression of sgRNA

CsVMV Promoter	443	*Cassava vein mosaic virus*	CsVMV promoter sequence
CsVMV 5' UTR	73	*Cassava vein mosaic virus*	Downstream of CsVMV promoter sequence
Replication Origins			
R2K oriV	618	*E. coli*	Plasmid origin of replication
(pUC) ORI	790	*E. coli*	Origin of replication
R2K TrfA	1482	*E. coli*	Encodes TrfA protein which binds to and activates R2K oriV
Terminators			
Nost	263	*Agrobacterium tumefaciens*	Termination sequence of the nopaline synthase gene, the function of this sequence is to signal the termination of the gene expression
35S terminator	204	Cauliflower mosaic virus	Terminates the transcription of the sequence immediately upstream of it
5' UTR			
TMV Omega	62	Tobacco Mosaic Virus	functions as a translational enhancer in plants

Part IV: Information relating 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 Brassicaceae family holds many agronomically important crop plants, including oilseed rape and cruciferous vegetables. Cruciferous vegetables can be found in a wide variety of morphotypes and have been consumed the world over for many years, having been bred and selected for their distinct flavour properties. The distinct flavour of Brassica vegetables is due to elevated levels of sulphur-containing metabolites, including the secondary metabolites known as glucosinolates. Glucosinolates are produced exclusively by plants of the order Brassicales (also referred to as Capparales) in order to deter herbivory (Mithen et al., 2010). The production of these sulphur-containing secondary metabolites is of economic significance due to their putative health-promoting abilities upon human consumption.

Myb28 has been repeatedly and independently well characterised as a vital regulator of aliphatic glucosinolate biosynthesis across the *Brassica* genus, including homologues in *Brassica napus* (Li et al., 2014), *Brassica juncea* (Augustine et al., 2013) and *Brassica rapa* (Kim et al., 2013; Seo et al., 2016). The role of Myb28 in production of potentially health-promoting compounds in broccoli has been part of a long-term research project at the Quadram Institute (formerly the Institute of Food Research) (Traka et al., 2013). It would be useful to understand the consequences of a knock out of this gene via CRISPR on sulphur metabolism and the production of glucosinolates. These plants have modified Myb28 sequence in order to remove gene function so that we may characterise the effect this genetic disruption has on the production of these sulphur compounds thereby obtaining an increased understanding of the effect of this gene in a commercial environment, similar to that of the broccoli lines.

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.

Small indels of a known glucosinolate biosynthesis regulator, Myb28, were introduced into the *Brassica oleracea* chromosomal DNA using CRISPR-Cas9 gene editing technology, as described in (Lawrenson et al., 2015). This region functions as the coding sequence of this gene, with the gene edits proposed to disrupt the translation of this gene, leading to a non-functional protein. This sequence change was characterised by PCR amplification of the gene region and subsequent Sanger sequencing.

These plants consist of subsequent generations of self-fertilised plants which underwent the *Agrobacterium*-mediated transformation to introduce the Cas9 and guide RNAs. PCR analysis and copy number analysis, in the progeny, of the NPTII promoter sequence described in Part III Section 12 has found no presence of the

transgene in these plants, suggesting that the insert is no longer present in these plants and has segregated out in the subsequent generations. However, there is the possibility of the backbone of the vector being integrated into the plants therefore absence of the vector components described in Part III Section 12, including the Cas9 sequences, will be confirmed using PCR techniques which are currently being developed.

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

(a) **information on the developmental expression of the insert during the lifecycle of the plant and methods used for its characterisation,**

(b) the parts of the plant where the insert is expressed, such as roots, stem or pollen.

Current data suggests the absence of the insert from the plants intended for the trial. However, should the presence of the Cas9 used for gene editing remain, it would likely be driven by the Cassava vein mosaic virus promoter and therefore be expressed in all tissue-types of the plant to varying degrees. Q-PCR results confirming the presence or absence of the Cas9 will be provided prior to planting, to the satisfaction of the regulator.

16. Information on how the genetically modified plant differs from the parental or recipient plant in the following respects -

(a) mode or modes and/or the rate of reproduction,

(b) dissemination,

(c) survivability.

As glucosinolates are used primarily for defence against insects and pathogens, it is possible that the reduction of these compounds would reduce the survivability of the genetically modified plant when compared to the parental/wild-type plant when placed in the field. Dissemination, modes and/or rate of reproduction should not be affected.

17. The genetic stability of the insert and phenotypic stability of the genetically modified plant.

The indels introduced by the Cas9 will remain fixed in the DNA of the genetically modified plant. Current data suggests an absence of the Cas9, which will be checked prior to release, to the satisfaction of the regulator.

18. Any change to the ability of the genetically modified plant to transfer genetic material to other organisms.

None known or expected.

19. Information on any toxic, allergenic or other harmful effects on human health arising from the genetic modification.

There are no known toxic, allergenic or harmful effects on human health that would potentially arise from the removal of glucosinolates from *Brassica oleracea*.

20. Information on the safety of the genetically modified plant to animal health, particularly regarding any toxic, allergenic or other harmful effects arising from the genetic modification, where the genetically modified plant is intended to be used in animal feeding stuffs.

There are no planned feeding studies of genetically modified plants with this trial.

21. The mechanism of interaction between the genetically modified plant and target organisms, if applicable.

Not applicable.

22. The potential changes in the interactions of the genetically modified plant with non-target organisms resulting from the genetic modification.

The genetic modification potentially leads to a reduction in glucosinolates, sulphur metabolites used by plants of this group to deter herbivory. It is possible that this genetic modification could lead to increased herbivory, as studies in the model plant *Arabidopsis thaliana* have found decreased larval weight gain of a generalist herbivore when over-expressing the gene edited in this study (Gigolashvili et al., 2007). However it is still unknown as to whether these plants will have low glucosinolate content and little data is available predicting herbivore interactions with these compounds in *Brassica oleracea* in the field.

23. The potential interactions with the abiotic environment.

Production of glucosinolates may be disrupted under low sulphur conditions, however this is unlikely to be affected during this trial as plants will be cultivated under standard commercial agricultural practices.

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

PCR using primers for the Myb28 gene-edited region followed by sequencing would detect presence of the modification in the coding region. PCR using primers

designed for components of the vector would give evidence for the presence of these components in the genome, however current data suggests that this is absent from these organisms.

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

Not applicable.

Part V: Information relating to the site of release

(Applications for consent to release only)

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

The plants will be released on an area of arable land no larger than 1000 metres squared located at the John Innes Centre (JIC, Ordnance Survey map grid reference TG 179 075). The land to be used is the area that was previously sown with GM potatoes under consent 10/R29/01 from 2010 – 2012.

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

The release site (Ordnance Survey map grid reference TG 179 075) is arable land located at the John Innes Centre (JIC); 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. With the exception of the surrounding *Brassica napus* guard crop*,* no *Brassica* plants will be grown within the accepted distance of 20 metres from the release site. Moreover, the plants in this trial will be removed prior to flowering, apart from a small number of plants for which their inflorescences will be secured within a pollen proof bag, therefore pollen release is highly unlikely.

28. Details of any sexually compatible wild relatives or cultivated plant species present at the release sites.

There are no sexually compatible wild Brassica relatives present on the release site. If present at all nearby the trial site, other related wild Brassica species will be limited to the boundary hedge/field margins of the trial site and thus will be separated by a distance of more than 20 metres from the genetically modified crop. Moreover, the plants in this trial will be removed prior to flowering, apart from a small number of plants for which their inflorescences will be secured within a pollen proof bag, therefore pollen release is highly unlikely.

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 4 km of the release site. The closest SSSI to the release site is Sweet Briar Road Meadows which is ~4 km away and is a series of unimproved wet meadows with permanent water-logging and thus very unlikely to host any Brassica plants.

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 determine the role of a gene, known as Myb28, in regulating sulphur metabolism, specifically the accumulation of aliphatic glucosinolates, in field-grown *Brassica oleracea*. Brassica plants of this type, when grown under glasshouse conditions, produce almost undetectable levels of these compounds, therefore this trial is required in order to better imitate the commercial interaction between these compounds and their environment and ultimately how this transcription factor Myb28 may mediate this interaction. Field evaluation of these traits allows for a better understanding on improvement of these crops in the future.

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

If consent is granted, the plants will be transplanted into the field in April/May with leaf and inflorescence material harvested in June/July from 2019 to 2021.

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

The plants are proposed to be sown and sequenced for presence of the desired mutation and any carry on of transgenic material from the vector, in the controlled glasshouse of the John Innes Centre, prior to individual transplantation to 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 staff from the John Innes Centre Field Experimentation team who carry out field work on the Norwich Research Park (NRP) site according to normal agricultural practices for *Brassica oleracea*. Ground

preparations will consist of existing grass being sprayed with herbicide to clear the ground. Compost will be applied if necessary and the ground will be prepared for planting using shallow cultivation. The plants will be covered by netted framework which will prevent damage from herbivores. Floret and leaf material will be harvested in June/July depending on weather conditions as these may affect flowering time. Material harvested will be used for gene expression and metabolite analysis. Following harvest, plants will be destroyed by autoclaving in order to prevent potential axillary flowering and pollen or seed release, apart from a small number of plants for which their inflorescences will be secured within a pollen proof bag. The plot will be monitored for presence of remaining *Brassica oleracea* during the remainder of the year and will be shallow tilled to remove weeds and encourage germination of any shed seed. Any Brassica plants identified will be removed by hand and destroyed by autoclaving within the John Innes Centre.

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

We estimate that the release will not exceed 50 plants.

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

35. A description of any precautions to -

(a) maintain the genetically modified plant at a distance from sexually compatible plant species, both wild relatives and crops.

The plants will be isolated from any other *Brassica* relatives, including other *Brassica* crops, by a distance of at least 20 metres. Should any species capable of cross-pollination with *Brassica oleracea* be identified, they will be destroyed by herbicide treatment (e.g. glyphosate). The release site will be routinely monitored for volunteers and any discovered will be destroyed. Post-harvest, the plot will be left fallow to allow identification of volunteers.

(b) any measures to minimise or prevent dispersal of any reproductive organ of the genetically modified plant (such as pollen, seeds, tuber).

Following harvest of the leaf and complete inflorescence for metabolites analysis, plants will be removed (including roots) and destroyed by autoclaving at the John Innes Centre with the exception of a small number of plants for which their inflorescences will be secured within a pollen proof bag. This minimises the risk of any pollen or seed release from potentially taking place.

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

Following harvest, the plot will be left fallow, monitored for remaining *Brassica oleracea* material during the remainder of the year and sprayed with a systemic broadleaf herbicide. Any *Brassica oleracea* identified will be destroyed by herbicide treatment (e.g. glyphosate) or removed by hand and destroyed by autoclaving as described below. The monitoring of the plot will be continued at monthly intervals by walking the trial site, in accordance with DEFRA guidance. During this time the plot will be left fallow to enable easy identification and removal of remaining *Brassica oleracea*. However, this is unlikely as *Brassica* is unable to clonally propagate and reproduces exclusively sexually, removal of the plants prior to flowering and bagging of remaining plants should remove the risk of seed being set and deposited into the soil.

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

All harvested material will be placed in sealed bags or containers and removed from site to an authorised waste disposal facility following analysis. Disposal will be carried out by incineration through our contractor SRCL.

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 genetically modified plants. The release site will be visited by trained laboratory personnel who are working on the project at no less than weekly intervals. Visits will usually occur more frequently. 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 will be inspected monthly and any *Brassica oleracea* identified will be immediately destroyed either by application of a systemic broadleaf herbicide or by hand pulling plants and digging out of the root systems. These will then be autoclaved within the John Innes Centre.

39. A description of any emergency plans.

At any time point post planting, should the release need to be terminated, any plant material will be sprayed with an appropriate systemic herbicide and roots dug up by fork and hand and transferred to an authorised waste facility for disposal by deep burying or incineration. 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 release site will be fenced to protect against animal damage and entry by unauthorised persons. The site will also be monitored by remote security cameras visible from the John Innes Centre (JIC) reception which is manned throughout the day by JIC reception staff and by security guards out of normal working hours.

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.

Methods for generating the transgenic lines are detailed in (Lawrenson et al., 2015).

Transformation of Brassica oleracea was performed as described in https://www.jic.ac.uk/app/uploads/2018/11/Brassica-Transformation.pdf updated from (Sparrow et al., 2006b)

Sequencing for presence of the mutation was conducted by PCR and sent for sequencing using the Eurofins Genomics Mix2Seq Platform.

Copy number of transgene detection conducted by Peter Isaac at iDNA Genetics Ltd (http://www.idnagenetics.com/index.php)

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