APPLICATION FOR CONSENT TO RELEASE A GMO – HIGHER PLANTS

PART A1: INFORMATION REQUIRED UNDER SCHEDULE 1 OF THE GENETICALY 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:
Rothamsted Research,
West Common, Harpenden
Hertfordshire,
AL5 2JQ
UK

2. The title of the project.

The synthesis and accumulation of omega-3 long chain polyunsaturated fatty acids and astaxanthin in *Camelina sativa*

PART II

Information relating to the parental or recipient plant

3. The full name of the plant –

(a) family name, Brassicaceae

(b) genus, *Camelina*

I species, *sativa*

(d) subspecies, *N/A*

I cultivar/breeding line, Celine

(f) common name. Gold-of-pleasure, false flax

4. Information concerning –

(a) the reproduction of the plant:

(i) the mode or modes of reproduction,

(ii) any specific factors affecting reproduction,

(iii) generation time; and

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

ai) Reproduction is sexual leading to formation of seeds. *C. sativa* is reported to be primarily, but not exclusively, self-pollinating. *C. sativa* is also pollinated by insects.

aii) Pollination, seed set and grain filling are dependent on temperature, weather conditions, agronomic practice and pressure applied by pests and disease.

aiii) The generation time is 85 -100 days, with seeds being sown in Spring.

b) It has been reported that *Camelina sativa* can intercross with other members of the Camelina genus, in particular *Camelina microcarpa* and *Camelina alyssum* (Seguin-Swartz et al., 2013). Within the larger Camelineae tribe, species such as *Arabidopsis lyrata*, *Capsella bursa pastoris* and *Neslia paniculata* are believed not to cross-hybridise with *C. sativa*, or result in viable seed (Julie-Galau et al., 2013). No cross pollination, either natural or forced, has been observed between *C. sativa* and members of the Brassica genus, such as *B. napus*, *B. juncea*, *B. rapa* and *B. nigra*. Artificial *in vitro* somatic hydrisiation between protoplasts of *C. sativa* and *B. napus*, *B. carinata* and *B. oleracea* has been reported, but with low success and/or sterile hybrids (http://www.inspection.gc.ca/plants/plants-with-novel-traits/applicants/directive-94-08/biology-documents/camelina-sativa-l-/eng/1330971423348/1330971509470 - this dossier also contains many useful details regarding the growth and cultivation of *C. sativa*)

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) & **b**) *C. sativa* is an annual species and survives from year to year only via seed production. Under agricultural practice, some mature seeds may fall from the plant prior to or at the time of harvest and not be collected. 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 over-wintering capacity of *C. sativa* grown in the UK, its tolerance of low temperatures and frost, photoperiod or dormancy.

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, but is more likely to be transmitted by insects such as bees. *C. sativa* is reported to be as attractive to bees as other species such as flax and canola. http://www.inspection.gc.ca/plants/plants-with-novel-traits/applicants/directive-94-08/biology-documents/camelina-sativa-l-/eng/1330971423348/1330971509470

Seed is usually retained by the plant within the seed capsules 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.

C. sativa originated in Europe, and was historically grown across South-Eastern Europe and South-Western Asia. It is a native species in many European countries, including Albania, Austria, Belarus, Belgium, Bulgaria, Czech Republic, Denmark, Estonia, Finland, France (including Corsica), Germany, Greece (including Crete), Hungary, Italy (including Sardinia, Sicily), Latvia, Lithuania, Moldova, The Netherlands, Norway, Poland, Romania, Russian Federation, Slovakia, Slovenia, Sweden, Switzerland, Ukraine and the United Kingdom.

C. sativa is grown as a crop in Canada and the Great Plains states (e.g. Montana, Nebraska) of the USA.

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.

C. sativa 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*) and pollen beetle (*Meligethes aeneus*). Other potential pests include cabbage root fly (*Delia radicum*) and the diamondback moth (*Plutella xylostella*).

Fungal pathogens are likely to include the following: *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.

Transgenic *C. sativa* plants were produced via *Agrobacterium*-mediated transformation ("floral dip") using published methods (Lu and Kang, 2008; Sayanova et al, 2012).

The Agrobacterium strain used was *Agrobacterium tumefaciens* strain GV3101 (Koncz and Schell, 1986).

11. The nature and source of the vector used.

The genes of interest were carried on a binary vectors pSUN2 or pRS-3GSeed (Ruiz-Lopez et al, 2012; Sayanova et al, 2012) both of which are derivatives of pBIN19 (Frisch et al, 1995).

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

Chemically synthesised gene sequences, codon-optimised for expression in *C. sativa*, were produced by GenScript Inc. NJ, USA and introduced into plant cells on complete binary plasmids by *Agrobacterium*-mediated transformation (see Table and notes below).

Element	Size	Donor Organism	Description and Intended Function
RB	24bp	*Agrobacterium tumefaciens*	T-DNA Right border
LB	23bp	*Agrobacterium tumefaciens*	T-DNA Left border
Seed-specific promoters			
USP	684bp	*Vicia faba*	Unknown Seed Protein Seed-specific promoter
NP	664bp	*Brassica napus*	Napin seed specific promoter
CNL	1064bp	*Linum usitatissimum*	2S seed storage protein (Conlinin) promoter
GLY	702bp	*Glycine max*	11S Seed storage protein (Glycinin) promoter
SBP	1800bp	*Arabidopsis*	Sucrose-binding protein promoter (seed-specific)
Omega-3 biosynthetic genes			
OtΔ6	1665bp	Synthetic	Encodes a fatty acid **Δ6-desaturase** from the marine picoalga ***O****streococcus* ***t****auri*

PSE1	873bp	Synthetic	Encodes an acyl-CoA-dependent **Δ6-elongase** from the moss ***Phys**comitr**e**lla patens*
TcΔ5	1320bp	Synthetic	Encodes a fatty acid **Δ5-desaturase** from the marine species ***T**hraustoc**h**ytrium*
Piw3	1086bp	Synthetic	Encodes a fatty acid **w3-desaturase** from ***P**hytophora **i**nfestans*
PsΔ12	1197bp	Synthetic	Encodes a fatty acid **Δ12-desaturase** FAD2 activity from ***P**hytophora **s**ojae*
OtElo5	903bp	Synthetic	Encodes an acyl-CoA dependent **Δ5-elongase** from the marine picoalgae ***O**streococcus **t**auri*
TpΔ4	1653bp	Synthetic	Encodes a fatty acid **Δ4-desaturase** from the marine diatom *Thalassiosira pseudonana*
O809Δ4	1480bp	Synthetic	Encodes a fatty acid **Δ4-desaturase** from the marine picoalgae *Ostreococcus* RCC809
Astaxanthin biosynthetic genes			
ZmPhys	1232bp	Synthetic	Encodes phytoene synthase from maize
HBFD1	1226bp	Synthetic	Encodes carotenoid 4-hydroxy-β-ring 4-dehydrogenase from *Adonis aestivalis*
CBFD2	687bp	Synthetic	Encodes carotenoid β-ring 4-dehydrogenase from *Adonis aestivalis*
Marker genes			
DsRed	684bp	Synthetic	Encodes a florescent protein from ***D**isco**s**oma* spp.
bar	548bp	Synthetic	Encodes phosphinothricin acetyl transferase, providing to resistance to Class H herbicides such as Basta ™, in which bialaphos is the active ingredient.
Regulatory elements			
pNOS	288bp	*Agrobacterium tumefaciens*	Nopaline synthase gene promoter

NOS*t*	256bp	*Agrobacterium tumefaciens*	Nopaline synthase gene terminator sequence
OCS*t*	192bp	*Agrobacterium tumefaciens*	octopine synthase gene terminator sequence
35S*t*	216bp	Cauliflower mosaic virus	35S transcript terminator sequence
CatpA*t*	235bp	*Arabidopsis thaliana*	Cathepsin A gene terminator sequence
Gly*t*	451bp	*Glycine max*	11S storage protein (Glycinin) terminator
E9*t*	558bp	*Arabidopsis thaliana*	Ubiquitin E9 ligase gene terminator sequence

PART IV

Information relating to the genetically modified plant

13. A description of the trait or traits and characteristics of the genetically modified plant which have been introduced or modified.

The omega-3 long chain polyunsaturated fatty acids (LC-PUFA) eicosapentaenoic acid (abbreviated to EPA; $20{:}5\Delta^{5,8,11,14,17}$) and docosahexaenoic acid (abbreviated to DHA; $22{:}6\Delta^{4,7,10,13,16,19}$) are components of fish oil, and known to help protect against cardiovascular disease. No higher plants contain EPA or DHA in their seed oils, although very many vegetable oils are rich in the simpler/shorter omega-3 α-linolenic acid (abbreviated to ALA; $18{:}3\Delta^{9,12,15}$). Through the seed-specific expression of algal and fungal genes involved in the biosynthesis of omega-3 LC-PUFAs, ALA is converted to EPA and thence DHA. This conversion only occurs in the seed, and results in a seed oil in which the fatty acid profile is modified. No alteration to fatty acid composition or lipid profile is observed in any other vegetative part of the plant.

The ketocarotenoid astaxanthin is found in a range of marine microorganisms, and the accumulation of this compound in marine foodwebs results in the distinctive pink pigmentation found in many organisms. Astaxanthin is also included in the diets of farmed fish such as salmon. Natural sources of astaxanthin include some marine species of krill and shrimp, and the microalga *Haematococcus pluvialis.* There are very few examples of higher plants which accumulate astaxanthin, the most notable example being the bright red flowers of the Adonis family. However, there are no reports of higher plants accumulating astaxanthin as a seed storage reserve. Astaxanthin biosynthetic genes from *Adonis aestivalis* were placed under the control of seed-specific promoters, resulting in the accumulation of this ketocarotenoid in *C. sativa* seeds. No accumulation of this compound was observed in any vegetative part of the plant.

Both omega-3 LC-PUFAs and astaxanthin are currently sourced from the marine environment. Thus, there is interest in producing new sources of these compounds to reduce reliance on oceanic stocks. Both compounds are used in aquaculture as feed ingredients.

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.

It is proposed to evaluate the performance of three individual events derived from the constructs listed below (Constructs **B7.2**, **DHA2015.1**, **ASX-A2**). In addition, a genetic cross between **ASX-A2** and **B7.2** will be evaluated, to determine the efficacy of stacking the individual traits associated with these constructs.

The identity of the various genes and regulatory elements is shown above in the Table in **Section 12**. The nature of the constructs and the specific event to be considered for release are described in detail below. For clarity, a simplified representation of the omega-3 long chain polyunsaturated fatty acid biosynthetic pathway is shown below. More details can also be found in Haslam et al 2013).

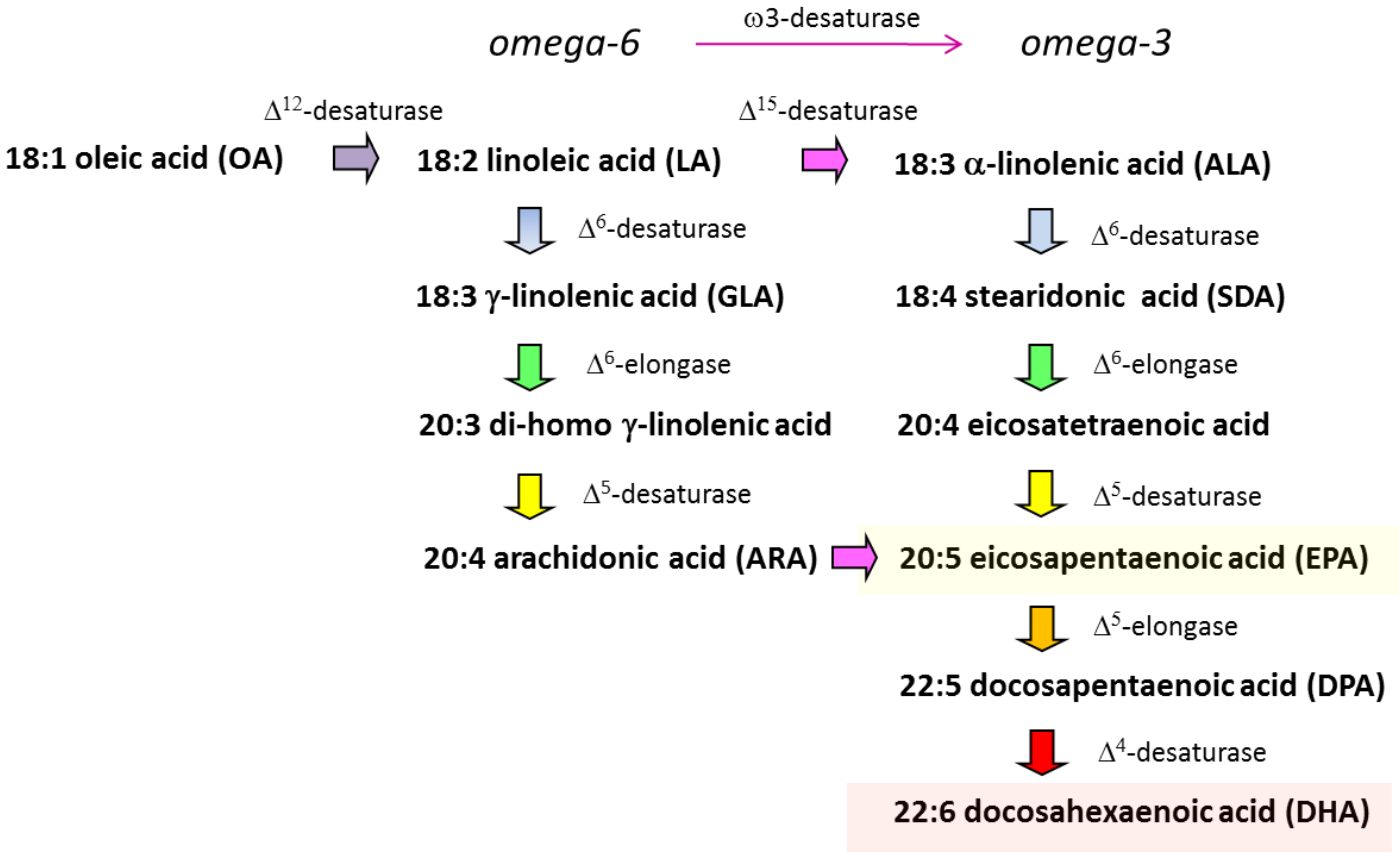
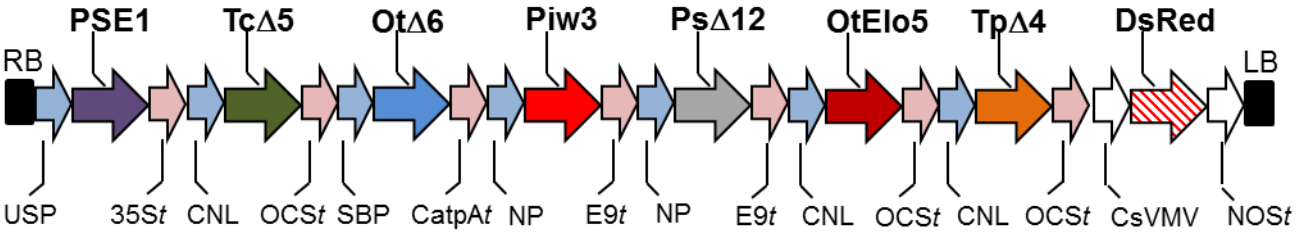


Fig 1. Schematic representation of long chain polyunsaturated fatty acid biosynthesis

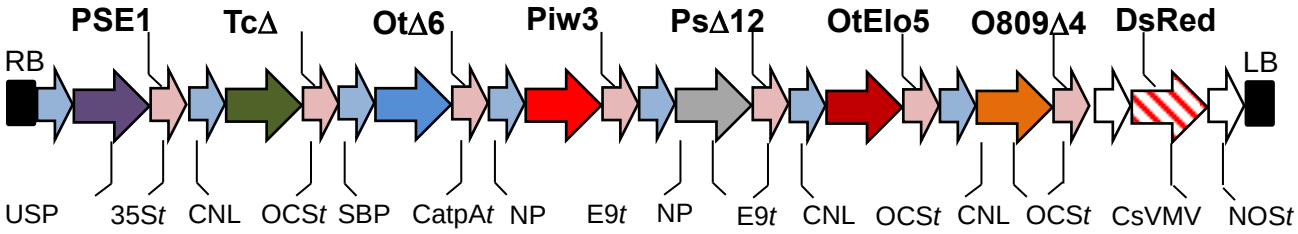
Constructs used to generate transgenic *C. sativa*

Omega-3 Long Chain Polyunsaturated Fatty Acids

Construct B7.2 (Size of T-DNA insert is 21,239 bp; event ID B7.2-4-1-6)



Construct DHA2015.1 (Size of T-DNA insert is 20,963 bp; event ID #20)



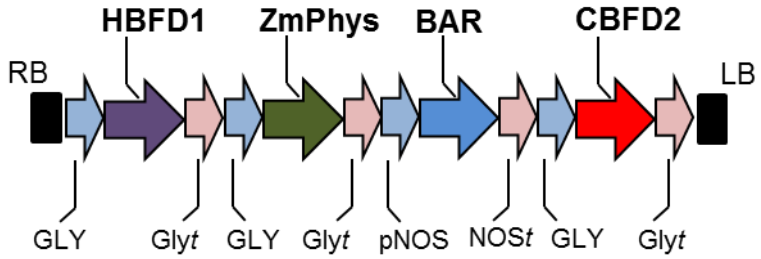
In **Construct B7.2**, seven heterologous genes under the control of seed-specific promoters were assembled to direct the synthesis of EPA and docosahexanoic acid (DHA) in the seeds of transgenic *C. sativa*. The heterologus pathway commenced by the Δ6-desaturation (**OtΔ6**) of endogenous fatty acids such as alpha-linolenic acid (ALA) and linoleic acid (LA). These Δ6-desaturation products (SDA, GLA) then undergo C2-elongation (**PSE1**) to the equivalent C20 fatty acids, which then receive a further double bond at the Δ5-postion by the Δ5-desaturase (**TcΔ5**). These products then undergo a second C2-elongation (**OtElo5**) to generate C22 polyunsaturated fatty acids, which are then substrate for the Δ4-desaturase () which is responsible for the direct synthesis of DHA (Fig. 1). In **Construct DHA2015.1**, the Δ4-desaturase was **O809Δ4** , replacing **TpΔ4.** In all other respects, the constructs were identical. Both constructs are stabley inherited in a Mendelian fashion, observed for T5 generations.

The accumulation of C20 and C22 omega-3 fatty acids such as EPA and DHA is enhanced by the activity of a ω3-desaturase (**Piw3**), which converst omega-6 fatty acids to the omega-3 form (Fig. 1). Additional substrate for **OtΔ6** was generated through the activity of the Δ12-desaturase (**PsΔ12**). All seven of these genes are synthetic, being codon-optimised from their native sequences (derived from the picoalgae ***O**streococcus **t**auri* or ***O**streococcus* RCC**809**, the moss ***P**hy**s**comitr**e**lla patens*, the Thraustochytriaceae ***T**hraustoc**h**ytrium*, the oomycetes ***P**hytophora **i**nfestans* and ***P**hytophora **s**ojae* and the diatom ***T**halassiosira **p**seudonana*) to that of a form that represents the codon-usage of dicotylendous plants, specifically the Cruciferae. Each synthetic coding sequence is regulated by an individual seed-specific promoter, and also defined by a transcription termination sequence. Within the T-DNA there is also the visual selectable marker, DsRed, which is constituitively expressed under the control of the casava vien mosiac virus promoter and nopaline synthase terminator. This T-DNA region has been introduced into transgenic *C. sativa* by *A. tumefaciens*-

mediated transformation, and primary transgenic events identified by the floresence of DsRed at 555nm. Indivual plants were self-pollinated, and homozygous progeny identifed on the basis of strong floresence and fatty acid profile, with homozygosity confirmed by the 100% presence of these transgene-derived traits in the next (selfed) generation.

Astaxanthin

Construct ASX-A2 (Size of T-DNA insert is 9,238 bp; event ID #7)



In Construct ASX-A2, three heterologous genes under the control of seed-specific promoters were assembled to direct the synthesis of the ketocarotenoid astaxanthin in the seeds of transgenic *C. sativa*. A phytoene synthase from maize (ZmPhys) was introduced to enhance the accumulation of phytoene (by the conversion of geranylgeranyl-diphosphate to phytoene) and subsequently to b-carotene, the latter to provide substrate for the carotenoid β-ring 4-dehydrogenase (CBFD2) from *Adonis aestivalis.* The product of that enzyme, a 4-hydroxy-β-ring, is then converted to astaxanthin by the 4-hydroxy-β-ring 4-dehydrogenase (HBFD1) from *Adonis aestivalis.* All three genes are synthetic, being codon-optimised from their native sequences (derived either from maize or *Adonis aestivalis*). Each synthetic coding sequence is regulated by an individual seed-specific promoter, and also defined by a transcription termination sequence. Within the T-DNA there is also the selectable marker, BAR, which is constituitively expressed under the control of the nopaline synthase promoter and terminator. This T-DNA region has been introduced into transgenic *C. sativa* by *A. tumefaciens*-mediated transformation, and primary transgenic events identified by their resistance to bialaphos (glufosinate-ammonium). To check for the presence of astaxanthin accumulation, astaxanthin was extracted from seeds with acetone and the absorbance of the extract was measured at 475 nm. This was compared to a standard curve constructed using commercially available astaxanthin. Seeds from lines which show astaxanthin accumulation were then sown on ½ M+S plates containing 300 ug/ml glufosinate-ammonium and survivors were transferred to soil. The traits encoding in the ASX-A2 construct are inherited in a Mendelian fashion indicative of a single insertion, and have been shown to be stable to the T5 generation.

Stack B7.2+ASX-A2 (B7ASX)

Homozygous plants containing **Construct B7.2** were used to cross-polinate plants containing **Construct ASX-2**, and the resulting seeds used to identify progeney which contained both the omega-3 trait and the astaxanthin trait. Plants from this initial cross were grown up and self-polinated, and the F2 seed collected. F2 seeds were screened for the PUFA trait by the fluorescence of DsRed at 555nm. DsRed positive seeds are then sown on ½ M+S plates containing 300 ug/ml glufosinate-ammonium to identify the individuals which were also carrying the ASX construct. Survivors of the herbicide treatment were transferred to soil allowed to develop as usual. Plants were bagged prior to flowering to prevent further crossing. F3 seeds were collected and screened as before, for the presence of both constructs. Seedlings dispaying both traits were transferred to soil and propagated as before to produce homozygous F4 seeds. The presence of the HBFD1 and the **TpΔ4** Δ4-desaturase were determined by PCR to confirm the presence of the two unlinked T-DNA inserts for the ASX and DHA traits, respectively, and shown to be stably inherited across 4 generations. The specific homozygous cross to be trailed has the ID #10.

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.

The transgenes encoding the omega-3 LC-PUFA and astaxanthin biosynthetic pathways are all under the control of seed-specific promoters which are maximally active during the mid-stage of seed development. Expression is not observed in any other vegetative tissue during the life cycle of the plant.

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.

Except for the accumulation of non-native omega-3 LC-PUFAs and ketocarotenoids such as astaxanthin in their seeds, the transgenic *C. sativa* plants are visually indistinguishable from the untransformed controls. No difference in seed set, seed size or germination was observed. No difference in fertility was observed. Vegetative performance of the transgenic plants was unaltered.

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

All plants expressing the transgenes are morphologically indistinguishable from untransformed controls. The inheritance of the transgene over 3+ generations follows normal rules of Mendelian genetics.

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 known to be associated with omega-3 long chain polyunsaturated fatty acids or astaxanthin and associated ketocarotenoids. The fatty acids are very widely consumed by humans, being recognised as important and beneficial nutrients, and astaxanthin is widely dispersed in the marine foodweb (it is the compound which gives salmon flesh the pinkish/orange hue). Astaxanthin is also consumed as a direct human dietary supplement and also has GRAS status in the USA.

The visual marker DsRed (Jach et al., 2001) encoding the red fluorescent protein from reef coral Discosoma sp was used in Construct **B7.2**. There are no known toxic, allergenic or harmful effects known to be associated with the DsRed protein.

The selectable marker *bar* (bialaphos resistance) encoding a phosphinothricin acetyl transferase; (PAT) activity from *Streptomyces,* which provides resistance to herbicides which act as inhibitors of glutamine synthase, a key enzyme in the nitrogen assimilation pathway of plants. The *bar* gene is present in **ASX-A2** and the derived cross **B7ASX**. There are no known toxic, allergenic or harmful effects known to be associated with the presence of this gene.

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 feeding studies planned associated 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.

There are no obvious mechanisms that could result in a change in behaviour of non-target organisms as a result of exposure to omega-3 long chain polyunsaturated fatty acids and/or ketocarotenoids, nor with the proteins associated with the marker genes (DsRed, *bar*). Thus, the likelihood of this potential hazard ever being realised is extremely low.

23. The potential interactions with the abiotic environment.

There are no known or predicted interactions associated with these traits and abiotic stress.

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

PCR using primers specific for recoded (i.e. unique) transgenes such as *O. tauri* Δ6-desaturase (**B7.2** and **DHA2015.1**) or the maize phytoene synthase (**ASX-A2**) can be used to detect GM Camelina. Similarly, in iterations **B7.2** and **DHA2015.1**, the presence of the florescent protein DsRed also acts a visual reporter on excitation at 555nm, whereas the presence of the *bar* gene and associated resistance to bialaphos is present in **ASX-A2**. In the crossed stack **B7ASX**, all of these traits are present.

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

Transgenic *C. sativa* containing a related construct for the seed-specific expression of EPA and DHA was previously approved in the UK for experimental field release (DEFRA Consent14/R8/01). That construct (Iteration C in 14/R8/01) varies only in the use of a different microalgal Δ4-desaturase, but in all other aspects, the transgene construct was identical – however, the levels of EPA and DHA present in the iterations to be tested in this application are higher than previously evaluated under the 14/R8/01 consent. Transgenic *C. sativa* containing Construct **ASX-A2** was recently approved in the US for experimental field release (APHIS Permit # 15-357-101r). However, no omega-3 LC-PUFA constructs are considered in that permit.

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 area for the proposed field trial, including controls, pollen barrier and spacing between GM plots will cover~50m x 50m (see plan in section 34).

We propose to carry out 2 trials in consecutive seasons and will avoid reusing the same plots. It will be sited in the farm at Rothamsted Research, Harpenden, UK and at grid reference TL 120130.

Enclosing the whole site will be a 2.4m high chain-link fence (with lockable double gates) and within that a second fence also with lockable gates to prevent the entry of rabbits and other large mammals including unauthorised humans.

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

The release site is an agricultural area forming part of an experimental farm. The flora and fauna are typical of agricultural land in the South East.

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

C. sativa is known to cross-pollinate with other close members of the Camelina tribe. Hybridisation with more distantly related Brassicaceae such as members of the Capsella tribe may be possible (Julie-Galau et al., 2013; Seguin-Swartz et al., 2013). In the cases of Camelina species which readily cross-hybridises with *C. sativa* (such as *C. alyssum*, *C. microcarpa*), there are no observed or reported cases of these species present on the Rothamsted farm (which is 330 ha in size). Querying the National Biodiversity Network database (www.nbt.org.uk) for the presence of *C. alyssum*, *C. microcarpa* and *C. rumelica,* returns no reports of these species being present within 15 km of the Rothamsted farm. Whilst potential cross-hybridising species such as *Capsella bursa-pastoris* are widely distributed across the UK and commonly found in Hertfordshire, the ability of *C. sativa* and *C. bursa-pastoris* to form viable offspring has experimentally been demonstrated to be very limited (Julie-Galau et al., 2013).

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

There are no protected areas near the trial site.

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 agronomic performance and seed oil yield of transgenic *C. sativa* plants that have been engineered to accumulate omega-3 long chain polyunsaturated fatty acids and/or ketocarotenoids in their seed oils. Specifically, we wish to examine the field performance of **DHA2015.1**, which display a superior EPA and DHA seed oil content to lines tested previously. Simultaneously, we will examine the performance of a genetic cross between an astaxanthin-accumulating line (**ASX-A2**) and an omega-3 line (**B7.2**). To ensure the appropriate comparisons are made, the parental lines (**ASX-A2, B7.2**) will be trialled alongside the stacked cross (**B7ASX**). Thus, individual events from four lines will be tested in both years – **ASX-A2**, **B7.2, B7ASX** and **DHA2015.1**.

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

The GM *C. sativa* will be planted consecutively for two years (2016-17). The plants will be sown in April/May and harvested in Aug/Sept.

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

Seeds will be drilled using conventional plot-scale farm equipment or hand-sowing.

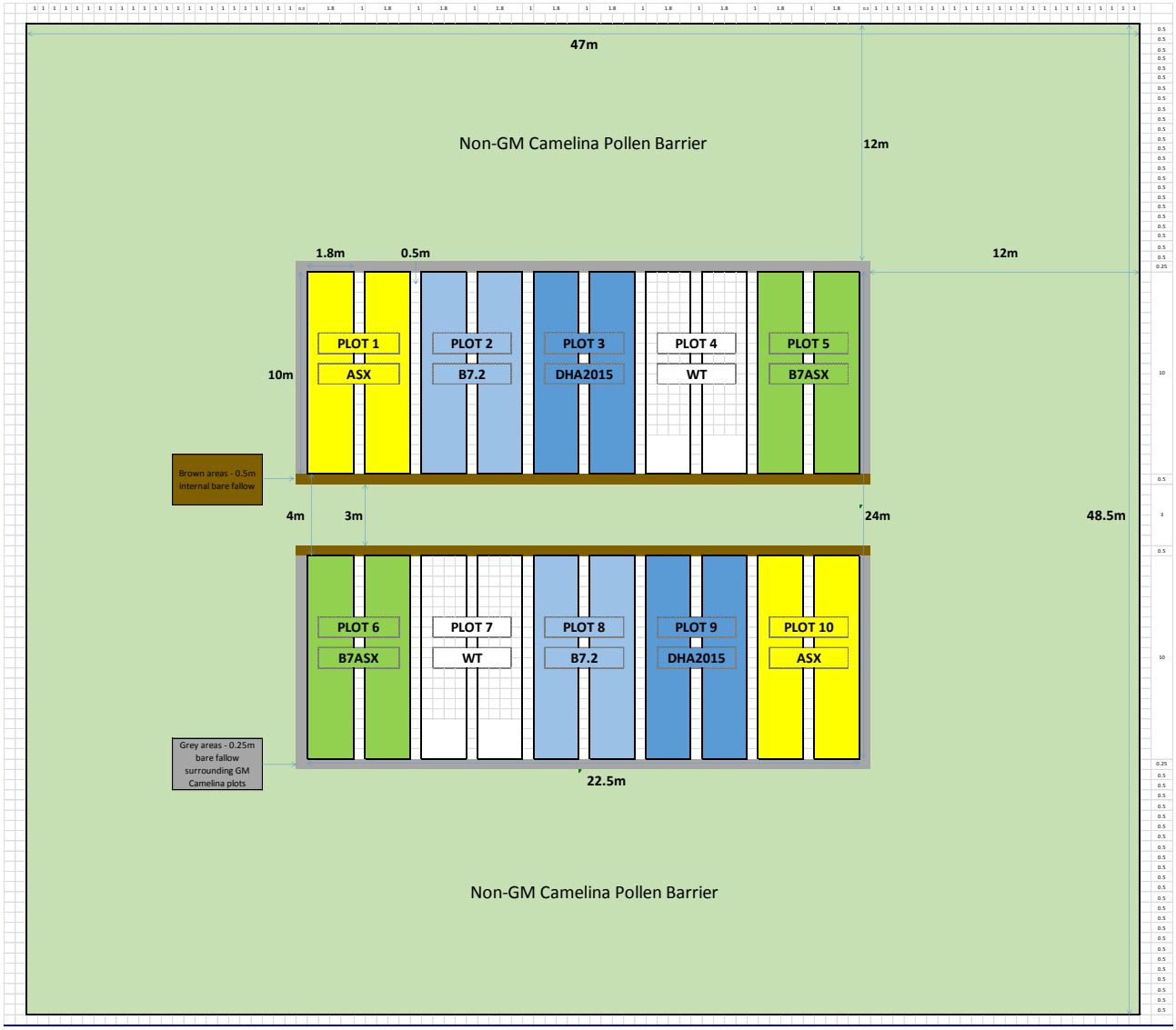
33. The method for preparing and managing the release site, prior to, during and after the release, including cultivation practices and harvesting methods.

The site will be prepared according to standard agronomic practices for spring Brassicaceae cultivation. The release will be monitored regularly during all stages of development and harvested just prior to full maturity. Some seeds from the GM and control plots will be conditioned, threshed and analysed in appropriate GM facilities to determine oil composition and oil quantity. All other material, including that from the pollen barrier will be harvested and disposed of by incineration or deep burial at a local authority-approved landfill site using an approved contractor. Transportation of waste materials will be in secure containers.

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

See trial design below (Fig. 2). GM plants will be sown in duplicated blocks made up of two strips of 1.8X 10m (36m^2 per block, thus 72m^2 per line). The outer pollen barrier will be sown with non-GM *C. sativa* of the same variety as the GM plots.

Sowing density will be to produce a stand density of less than 300 plants per m^2 to accommodate any variation in germination rates. It is proposed to grow 4 GM *C. sativa* lines, with the trial design including two identical control (WT) plots of two strips (please see Section 30 above for further details). Thus, the total area of GM *C. sativa* would be 16 x 1.8 X 10m (288m^2), for both years.



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.

There are no sexually compatible species present within the Rothamsted estate.

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

The trial has a 12m strip of non-GM *C. sativa* to function as a pollen barrier. The drills will be filled on the trial area and will be thoroughly cleaned before leaving the trial area. To minimise the possibility of seed loss, the plants will be harvested just prior to full maturity. All straw will be chopped and left on site.

Netting will be fixed over the trial plots to keep birds out when the *C. sativa* is in flower. Other measures (suspending wires across the area to provide bird scaring as well as gas guns and hawk kites) will be used to keep out birds for the rest of the season. At drilling all care will be taken to ensure that no seed remains on the surface after drilling because this encourages pigeons in to try and find seeds. Therefore the risk is minimal at sowing and the bird scaring measures specified above will be enough to mitigate the risk. Appropriate husbandry steps will be taken to minimise the potential for seed dispersal by molluscs such as slugs.

Pollen dispersal be minimised through the placing of wildtype *C. sativa* on the external strip of the experimental plot – this will serve as a pollen-trap for pollen released from the GM *C. sativa*. In addition, the entire site is contained by two chain-link fences, which also serve as physical barriers to impede foraging bees. To further mitigate against pollen dispersal by insects, the GM *C. sativa* will be covered with netting (0.25mm fine mesh) during the flowering period to exclude any insects which could act as pollen carriers.

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

The trial will receive standard farm practise as regard to herbicide, fungicides and nitrogen in conjunction with the scientific co-ordinator. The site will be regularly monitored from sowing to harvest and during the following cropping year.

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

At harvest, all seeds from the GM plots will be collected with a plot combine to obtain yield measurements. The seed sampled will be analysed on site at Rothamsted Research, all samples taken from the field will be closely monitored and records kept of weights and movements of seeds and straw. All samples removed from the trial site will eventually be destroyed by an approved technique. The remainder of the site will be then be harvested by the plot combine. The grain obtained will be disposed to deep landfill using an approved contractor. The plot combine will be cleaned in an uncultivated region of the fenced area prior to leaving the site so that all traces of GM plant material will remain in the trial area. The trial area will remain in stubble for the following year to enable monitoring of volunteers and a broad spectrum herbicide such as glyphosate will be applied as required.

38. A description of monitoring plans and techniques.

The site will be monitored regularly (at least weekly) during the growing period (May-Aug) and after the termination of the trial during the following year. Records will be kept of each visit.

39. A description of any emergency plans.

In the unlikely event that the integrity of the site is seriously compromised, the trial will be terminated and all plants, (including GM and control plots and pollen barrier) will be destroyed using a suitable herbicide or harvesting as deemed appropriate. All harvested material will be removed from the site and disposed of by incineration or deep burial at a local authority-approved landfill site using an approved contractor. Transportation of waste materials will be in secure containers. The phone numbers of all key staff will be available to site security and farm.

40. Methods and procedures to protect the site.

The Hertfordshire Constabulary have been notified that we intend to carry out GM field trials at Rothamsted Research in the near future and we have started discussions with them as regard to site security. We have previously fenced a total area of approximately 3 hectares with a 2.4m high chain-link fence (with lockable double gates) and to have movement-activated cameras and a manned security presence. It is within these fixings that the GM *C. sativa* will be planted.

PART VIII

Information on methodology

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

1. DNA synthesis was carried out by GenScript Inc. USA (http://www.genscript.com/index.html).

2. Standard molecular biology reagents and methods were used following Sambrook et al, (1989).

3. *C. sativa* transformation via floral infiltration was performed using *Agrobacterium* as described in Lu and Kang, (2008). This method avoids the use of any tissue culture steps in the generation of the transgenic *C. sativa* lines.

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PART A2: DATA OR RESULTS FROM ANY PREVIOUS RELEASES OF THE GMO

The constructs described in this application have not previously been evaluated in the field.

PART A3: DETAILS OF PREVIOUS APPLICATIONS FOR RELEASE

Rothamsted Research has received consents to release GM wheat (e.g. 97/R8/3, 01/R8/4 and 11/R8/01 and more recently GM *C. sativa* (14/R8/01)

PART A4: RISK ASSESSMENT AND A STATEMENT ON RISK EVALUATION

Summary

Observations on the general plant morphology of glasshouse-grown plants, timing of flowering, fertility, seed shape and germination show that the GM *C. sativa* events are indistinguishable from their non-GM equivalents.

Where applicable, the gene donor organisms are not known to be pathogenic or allergenic to humans, and none of the genes under investigation, or the selectable or visual 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 *C. sativa* plants will not be consumed by humans.

The probability of *C. sativa* seeds escaping from the trial site or the transfer of inserted characteristics to sexually-compatible species outside the trial area is estimated as very low. *C. sativa* seeds are moderate in size and not normally dispersed by wind. Management measures including netting when the *C. sativa* is in flower and the use of gas guns and hawk kites will be employed to mitigate the risk of seed removal by birds. Management procedures to minimise the spread of seeds or pollen (such as insect-excluding netting) will further reduce the probability of these events occurring. There will be no compatible species grown for 1000 meters from the boundary of the site and no sexually-compatible wild relatives of *C. sativa* exist in the vicinity of the Rothamsted farm. In the unlikely event of a hybrid being generated, the presence of EPA+DHA and/or astaxanthin in the seed oil of any such progeny would not convey a selectable advantage and most likely the omega-3 and/or ketocarotenoid trait would not be retained. This is equally true for the marker gene *bar* since no selective bialaphos-containing herbicides will be used in the management of this field trial.

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 genes nor the marker genes would be expected to confer a selective advantage in the field environment under consideration. The genes introduced in *C. sativa* have been inserted via *Agrobacterium tumefaciens*-mediated gene transfer.

We estimate the likelihood of horizontal gene transfer as low and the consequences were it to occur, as negligible. The area proposed to be planted with GMOs is small and temporary (lasting between 4 and 5 months).

The overall risk of harm to human health or the environmental arising from this trial is assessed as very low.

Detailed evaluation of hazards, magnitude of exposure and management strategies to minimise risk.

We adopted a classic six-step process of risk assessment. Systematic identification of all potential hazards arising from this field trial; evaluation of hazard-realisation in the specific field-trial environment; potential for harm; frequency of exposure; mitigation of risk by appropriate management and finally, an estimate of the overall risk.

Step1: ***Potential hazards which may be caused by the characteristics of the novel plant***	**Step 2:** ***Evaluation of how above hazards could be realised in the receiving environments***	**Step 3:** ***Evaluation the magnitude of harm caused by each hazard if realised***	**Step 4:** ***Estimation of how likely/often each hazard will be realised as harm***	**Step 5:** ***Modification of management strategies to obtain lowest possible risks from the deliberate release***	**Step 6:** ***Overall estimate of risk caused by the release***
Increased invasiveness in natural habitats or persistence in agricultural habitats due to inserted trait.	Increased invasiveness may arise from intended or unintended effects of the genetic modification that resulted in *C. sativa* plants with a more 'weedy' habit that are better able to establish and thrive in uncultivated environments or to persist in agricultural habitats.	*C. sativa* is an annual species that requires active management to out-compete more weedy plants. Left unmanaged, it does not establish well in nature and thus has a low base line of invasiveness and persistence. Even if intended or unintended effects of the genetic modification resulted in major changes in invasiveness or persistence, it is considered that this would not result in significant environmental harm for agricultural or unmanaged ecosystems. *C. sativa* is a benign plant that can be easily managed by cultivation or specific herbicides. The magnitude of harm if the hazard was realised is considered to be very small.	It is highly unlikely that intended or unintended effects of the genetic modification will result in major changes in invasiveness or persistence. If it were to occur, this hazard would be realised only if seeds or pollen possessing genes encoding these traits were to spread from the trial site and successfully become established elsewhere. This is very unlikely as there are no wild or cultivated relatives of *C. sativa* that can cross-hybridise and produce viable seeds. Seed removal from the site will be rigorously managed (see step 5). The chances of modified *C. sativa* plants establishing themselves outside the trial site are negligible.	Harvested seeds will be transported from the site in sealed containers. Machinery will be cleaned thoroughly prior to removal from the site. No *C. sativa* will be cultivated for at least 1000m surrounding the trial so it will be easy to see any *C. sativa* plants in the surrounding area. Appropriate physical barriers and/or deterrents will be employed to minimise access by large mammals and birds.	Overall risk is negligible.
Increased invasiveness in natural habitats or persistence in	Increased invasiveness may arise from the genetic modification in the specific	The *bar* marker gene present in two of *C. sativa* lines described in this application provides tolerance of the broad spectrum herbicide bialaphos (also known as glufosinate). The presence of this	The selectable advantage provided by the bar gene is only realised when the plant is exposed to the specific Class H herbicide. In the absence of this	No positive selection for the *bar* gene will be applied to the trial site, unless in the form of a closely controlled plot-size experiment to	Overall risk is very low.

agricultural habitats due presence of the selectable marker gene (*bar*)	cases where the selectable marker gene (*bar*) is included in the transgene cassette (present in Construct ASX-A2 and the derived cross B7ASX), resulting in *C. sativa* plants that are better able to resist specific herbicides	transgene could provide a selectable advantage to the GMO	selection pressure, there is no obvious advantage conferred by the presence of this gene. Monitoring and management measures will be carried out to ensure that no plants or seeds are removed from the trial site in anything other than controlled conditions. We estimate that the potential hazard associated with the presence of the *bar* gene is low.	evaluate the efficacy of this trait. No Class H herbicides (to which bar confers resistance) will be used in the general management of the trial	
Selective advantage or disadvantage conferred to other sexually compatible plant species.	Selective advantage or disadvantage may result from the intended traits (improved oil composition) or as a result of unintended effects of the genetic modification. These hazards could be realised in the receiving environment via dispersal of GM seeds from trial site to the surrounding environment or via out-crossing to sexually-compatible species outside trial site.	We anticipate that the conferred trait of improved oil composition will provide little or no change in selective advantage compared to other factors determining a plants ability to survive in unmanaged ecosystems. This is equally true for the visual marker protein DsRed, and also for the *bar* resistance marker, since in the latter case, no herbicides targeting the inhibition of glutamine synthetase will be used.	This potential hazard would be realised only if seeds or pollen possessing genes encoding these traits were to spread from the trial site and successfully become established in environments were the appropriate selection pressures were present. This is very unlikely as there are no sexually compatible species for out-crossing for at least 1000m from the trial site. Seed removal from the site will be rigorously managed. The frequency of this potential hazard resulting in environmental harm is very low.	Harvested seeds will be transported from the site in sealed containers. Machinery will be cleaned thoroughly prior to removal from the site. There is a buffer zone to minimize the spread of pollen, and also an insect-proof fine mesh net will be placed around the GM crop when it is flowering. Surrounding the trial site is an 1000 metre area in which no *C. sativa* will be grown Appropriate physical barriers and/or deterrents will be employed to minimise access by large mammals and birds. No herbicides of the H class will be used	Overall risk is very low.

Potential environmental impact due to interactions between the novel plant and other organisms	Omega-3 long chain polyunsaturated fatty acids and/or ketocarotenoids may illicit a change in behaviour of other organisms.	There are no obvious mechanisms that could result in a change in behaviour of non-target organisms as a result of exposure to omega-3 long chain polyunsaturated fatty acids and/or ketocarotenoids. Thus, the likelihood that this potential hazard ever being realised is extremely low.	Many organisms will encounter the modified *C. sativa* plants in the field trial.	Management practices will be put into place to minimise the contact of birds and mammals (eg bird kites etc). However the hazard is purely hypothetical and highly unlikely ever to be realised.	Overall risk is very low.
Potential effect on human or animal health due to introduced omega-3 long chain polyunsaturated fatty acids or ketocarotenoids	By contact or ingestion of GM plant material.	Omega-3 long chain polyunsaturated fatty acids are essential components of most vertebrates' diet, with these fatty acids widely recognised as being health-beneficial. They are very widely represented in the human food chain, without any reported negative effects. This is equally true to ketocarotenoids. Both compounds are present in natural food webs and do not appear to interact in a synergistic fashion	Some contact between the GM plants and humans or animals is expected. People operating farm machinery and scientists working in the trial site will come into physical contact with the plants. Small mammals such as mice, invertebrates and birds may also come into contact and/or ingest plant material.	No plant material from the trial will enter the food or animal feed chain. Appropriate physical barriers and/or deterrents will be employed to minimise access by large mammals and birds. Machinery will be cleaned before being removed from the trial site	Overall risk is very low.
Unexpected interactions between omega-3 long chain polyunsaturated fatty acids and ketocarotenoids	Toxic or unpredicted interactions between the omega-3 fatty acids and ketocarotenoids as a result of their co-expression in the seeds of the GMO	The magnitude of harm resulting from such an interaction is low. This would occur only in the seeds of the stacked lines, and if generating a toxic or deleterious outcome, might be expected to not result in viable seed. Both compounds (omega-3s, ketocarotenoids) already co-exist in both natural and artificial (i.e. farming) foodwebs, and no known examples of such hazardous interactions are known	If such an interaction was to generate a hazardous or toxic outcome, it is unlikely viable seeds (and hence plants) would have been recovered. However, it is possible such a hazard would only be realised through exposure or ingestion to the two compounds.	No plat material from the trial will enter the feed or food chain. Human and/or animal exposure will minimized and monitored via the appropriate management practices	Overall risk is very low.

Potential effects on human or animal health due to horizontal gene transfer of recombinant DNA	By contact, ingestion or infection with bacteria that had received recombinant DNA via horizontal gene transfer.	The magnitude of harm caused by contact, ingestion or infection with bacteria that had received the recombinant DNA via horizontal gene transfer is low. The introduced genes are not expected to be expressed in bacteria and would have no safety concern if they were.	The rate of horizontal gene transfer from genetically modified plants to other species is accepted to be extremely low. The absence of plasmid backbone sequence and origins of replication which are derived from E. coli and *Agrobacterium tumefaciens*, decrease the chances of homologous recombination between plant and microbial DNA in the soil. If recombinant DNA were to move by horizontal transfer to soil bacteria, it is extremely unlikely to alter their survivability or pathogenicity. The area proposed to be planted with GMOs is small <300m^2 and temporary (lasting <5 months/year for two years).	No plant material from the trial will enter the food or animal feed chain.	Overall risk is very low.
Consideration of the risk of horizontal gene transfer into wild-type Agrobacterium species in the soil that could infect and transfer DNA to other plant species including risks associated with expression of the genes.	By DNA released from decomposing plant material being taken up into the T-DNA of wild-type Agrobacterium and the subsequent expression of functional cassettes in other plants after natural transformation by *Agrobacterium*.	In the very unlikely event that functional expression cassettes were horizontally transferred into soil *Agrobacterium* cells and then somehow expressed in newly transformed plant cells, it is possible that this may alter the FA profile of the transformed cells in these plants.	Horizontal gene transfer between plants and wild-type *Agrobacterium* species, and the subsequent infection of other plant species with recombinant DNA is considered an exceedingly small risk. Although transformation of wild type *Agrobacterium tumefaciens* has been reported in laboratory experiments using pre-inoculated sterile soil and high concentrations of circular Ti plasmid with appropriate antibiotic selection (Demaneche et al 2001), no such demonstration has been reported in the field or with	This risk will be managed by minimising the seeds and other above-ground plant biomass left in the soil.	The risk of this is extremely low

			linearised plant DNA with or without selection. Even in optimised laboratory conditions, electroporation or freeze-thaw methods are required to effectively transform *Agrobacterium* spp (Holsters 1975, Mattanovich et al 1989). It is considered highly unlikely that free DNA liberated by degradation of GM plant roots in the soil would become stabilised in wild-type *Agrobacterium* and capable of autonomous replication. This could theoretically occur if the transgene insert liberated by decomposing roots was taken up by wild type *Agrobacterium* either as an intact plasmid or as a DNA fragment and subsequently incorporated into the resident Ti plasmid by for instance, homologous recombination. The former would stabilise only if the host *Agrobacterium* cell shared the same IncR compatibility group as the pSa origin of the transgene vector used in this trial.		
Potential effects on biogeochemical processes (changes in soil decomposition of organic material	Changes in biogeochemical processes may result from unintended changes in the modified plants or from unintended changes in soil	The magnitude of harm is estimated to be extremely low. Biogeochemical processes are not expected to be affected by the cultivation of the genetically modified plants.	The frequency of changes to biogeochemical processes is considered to be very low. The maximum area proposed to be planted with GMOs is small <300m^2 and temporary (lasting <5 months/year for two years).	None.	It is very unlikely that changes in biogeochemical processes would occur

	microbes due to horizontal transfer of DNA.				

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PART A5: ASSESSMENT OF COMMERCIAL OR CONFIDENTIALITY OF INFORMATION CONTAINED IN THIS APPLICATION.
Identify clearly any information that is considered to be commercially confidential. A clear justification for keeping information confidential must be given.

This is publically funded research and has no associated commercial confidentiality considerations.

PART A6: STATEMENT ON WHETHER DETAILED INFORMATION ON THE DESCRIPTION OF THE GMO AND THE PURPOSE OF RELEASE HAS BEEN PUBLISHED
Make a clear statement on whether a detailed description of the GMO and the purpose of the release have been published, and the bibliographic reference for any information so published. This is intended to assist with the protection of the applicant's intellectual property rights, which may be affected by the prior publication of certain detailed information, e.g. by its inclusion on the public register.

Previous iterations of the omega-3 constructs have previously been published in peer-reviewed journals, including data from year one the field trial covered under R8/14/01 consent

Ruiz-Lopez N, Haslam RP, Napier JA, Sayanova O. (2014) Successful high-level accumulation of fish oil omega-3 long-chain polyunsaturated fatty acids in a transgenic oilseed crop. *Plant J*. 77(2): 198-208. doi: 10.1111/tpj.12378.

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