Application to FSANZ for the Inclusion of wheat event IND-ØØ412-7 with increased tolerance to environmental stresses in Standard 1.5.2 Food Produced Using Gene Technology

Submitting Company:
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Submitted By:

Submitted On: 4th June 2021
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OECD Unique identifier:
IND-ØØ412-7

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Part 1 General Requirements (3.1.1)

A. Executive Summary

Trigall Genetics is a Uruguay-based joint venture between Bioceres Crop Solutions and Florimond Desprez. Trigall Genetics has developed a genetically modified wheat line using the sunflower *HaHB4* gene to confer increased tolerance to environmental stresses avoiding reduction of crop yield. The HAHB4 protein belongs to the HD-Zip family of transcription factors, characterised by the presence of two functional domains: the homeodomain (HD), responsible for DNA binding, and a leucine zipper motif (LZ) involved in protein-protein interaction and dimerisation. The wheat event described in this application has the unique OECD code: IND-ØØ412-7 and is referred to as 'HB4 wheat' in this submission.

HB4 wheat was developed using particle bombardment by co-transforming the wheat variety Cadenza with the plasmids *pIND4-HB4* and *pIND4-Bar*. The selected event (IND-ØØ412-7) has been field evaluated over several growing seasons in Argentina with data supporting the conclusion that the *HaHB4* gene confers increased tolerance to environmental stresses that reduce crop yields, and that wheat event IND-ØØ412-7 also exhibits tolerance to glufosinate-based herbicides.

Molecular characterisation of the event was performed to determine the number of copies, arrangement, and stability of the inserted DNA. Molecular analysis shows a complex integration structure. Two inserts (within a single locus) are present in wheat event IND-ØØ412-7, and the complete nucleotide sequence shows there is one complete copy of *HaHB4* gene and three copies of the *bar* gene, with the respective regulatory elements in the correct positions. Other genetic elements contained in the insertions are incomplete and/or non-functional copies of genes and genetic elements from the vectors used in the transformation.

Field trials were undertaken with wheat event IND-ØØ412-7 to compare agronomic performance and biosafety with the conventional variety and other cultivated varieties used as controls. Results from these trials confirmed no changes were observed in wheat event IND-ØØ412-7 that could have an impact on the environment. Stability of the genetic modification was assessed and confirm that the HB4 trait is stably inherited and conforms to Mendelian segregation principles.

Compositional analysis was performed following the OECD Consensus Document recommendations for wheat (OECD, 1999a, 2003) including treatments with the herbicide glufosinate (OEDC, 1999b). Comparison of nutritional and anti-nutritional compounds showed no biologically relevant differences exist that could result in increased harm to humans or other non-target organisms. Analysis of the HAHB4 and PAT proteins as well as putative polypeptides produced from the inserted DNA indicated there are no sequences with significant homology to known allergens or toxins in HB4 wheat.

Analysis of the HB4 wheat has not revealed any biologically relevant differences compared to the conventional variety, except for the intended tolerance to abiotic stress and herbicide tolerance. Collectively, results of the molecular characterisation, agronomic assessment, and composition analysis support this application for amendment to the *Australia New Zealand Food Standards Code* to allow inclusion of HB4 wheat in **Standard 1.5.2**-*Food Produced Using Gene Technology*.

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B. Applicant Details

(a)	Applicant's name/s	
(b)	Company/organisation name	Trigall Genetics
(c)	Address (street and postal)	c/o Ocampo 210 bis, 2000 Rosario, Santa Fe, Argentina
(d)	Telephone number	
(e)	Email address	
(f)	Nature of the applicant's business	Trigall Genetics is a Uruguay-based joint venture between Bioceres Crop Solutions and Florimond Desprez
(g)	Details of other individuals, companies or organisations associated with the application	Or

C. Purpose of the Application

This application seeks to amend the *Australia New Zealand Food Standards Code* to allow for the inclusion of wheat event IND-ØØ412-7 in **Standard 1.5.2**-*Food Produced Using Gene Technology*.

Trigall Genetics has developed and evaluated wheat events that have increased yield opportunity under conditions of environmental stress. The wheat event described in this application has the unique OECD code: IND-ØØ412-7 and is referred to as 'HB4 wheat' in this submission.

Currently, Trigall Genetics does not intend to import HB4 wheat into Australia or New Zealand for food consumption. The primary aim of this application is to obtain a food safety approval to protect international trade. Trigall Genetics is, however, exploring opportunities to introgress the HB4 trait into Australian wheat germplasm and would seek to obtain import approval from the Department of Agriculture, Water and the Environment and relevant cultivation approvals through other regulatory agencies such as the Office of the Gene Technology Regulator (OGTR) and the Australian Pesticides and Veterinary Medicines Authority (APVMA). This submission is consistent with Trigall Genetics commitment to global stewardship, adhering to industry best practice by obtaining regulatory approvals in production and import markets.

D. Justification for the Application

Trigall Genetics has developed a new wheat event, IND-ØØ412-7. The new wheat event was created using the sunflower *HaHB4* gene that confers increased yield opportunity under conditions of environmental (abiotic) stress. The event also contains the herbicide tolerance *bar* gene from *Streptomyces hygroscopicus*, expressing the glufosinate-inactivating enzyme phosphinothricin N-acetyl transferase (PAT).

Globally, wheat is the largest food crop in terms of area allocation (nearly 25% of global arable land) and is the second most produced cereal crop after maize (Velu and Singh, 2013). Wheat accounts for 56% of the global coarse grain production and is the staple food for more than one third of the world's population providing more calories and protein in the human diet than any other crop (Curtis, 2002; IDRC, 2010).

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World wheat production is forecast to increase slightly in 2019–20 to reach a record high of around 768 million tonnes in 2020–21 (ABARES, 2020). Consistent with this increase, Australian wheat production is forecast to more than double in 2020–21 to around 31 million tonnes.

The major global wheat producers forecast for 2020–21 include China (136 Mt), the European Union (125 Mt) the Black Sea Region[1] (121 Mt), India (108 Mt) and the United States (50 Mt) (ABARES, 2020).

Drought is the most significant environmental stress which limits crop productivity around the world. Low water availability at critical stages of crop development leads to great yield losses (Duque et al., 2013). ABARES research has shown that changes in climate conditions over the last 20 years have had an adverse effect on the productivity of Australian cropping farms (Hughes et al. 2017). Similarly, New Zealand has experienced several major droughts during the last decades, leading to significant agricultural production losses (Pourzand and Noy 2019).

It is predicted that the shift in climate toward higher temperatures and altered rainfall patterns (predominantly drier) are expected to lead to more frequent and intense drought conditions. As such, tolerance to drought stress is a highly desired goal of wheat genetic improvement and significant efforts are being made to develop wheat varieties with drought tolerance through conventional breeding (Vinocur and Altman, 2005; Witcombe et al., 2008; Pfeiffer et al., 2005; Gupta et al., 2012; Velu and Singh, 2013; IWYP, 2016). However, none of these approaches have resulted in market ready products. This is largely due to technical challenges associated with two main factors:

1. Drought tolerance is a complex trait that is controlled by many genes (Naeem et al., 2015).
2. The wheat genome is extremely long (40 times longer than rice and 5 times as long as the human genome) and complex consisting of three distinct sub-genomes with many repetitive sequences (IWGSC 2018).

To address these challenges new approaches have been required, including genome wide selection (Juliana et al., 2020) and genetic engineering (Shinozaki et al., 2003; Wang et al., 2003; Wang et al., 2016; Verma and Deepti, 2016).

Members of the HD-Zip family of transcription factors (TFs), unique to plants, have been shown to be involved in regulating the response of plants to environmental stress (Schena and Davis, 1992). Expression of genes of the HD-Zip subfamily I is regulated by external factors such as drought, extreme temperatures, osmotic stresses, and light conditions (Ariel et al., 2007; Chan, 2009). The *HaHB4* (*Helianthus annuus* homeobox 4) gene is a member of the HD-Zip sub-family I, coding for the sunflower transcription factor HAHB4 (González et al., 2020). The introduction of *HaHB4* gene in wheat event IND-ØØ412-7 led to the drought stress tolerance phenotype. Phenotypic and field performance selection of several *HaHB4*-containing lines allowed the development of a transgenic wheat (termed IND-ØØ412-7), which was shown to provide an increased yield opportunity under conditions of environmental stress.

E. Information to Support the Application

This application consists of 2 parts containing information in accordance with the following checklists:

- Part 1: General requirements (3.1.1)
- Part 2: Foods produced using gene technology (3.5.1) main document, Part 2: Specific Data Requirements for Safety Assessment.

[1] Black Sea Region: Kazakhstan, Russian Federation and Ukraine

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F. Assessment Procedure

Trigall Genetics is anticipating that this application will be considered under the **General Procedure** for Administrative Assessment process by Food Standards Australia New Zealand.

G. Confidential Commercial Information (CCI)

Confidential Commercial Information (CCI) has been included in this submission document. A separate expurgated copy of this application is also provided.

Specific information that is considered confidential is highlighted in this submission. Specifically:

- Page 40: Identifies the location of the HB4 wheat insertion within the wheat genome
- Figure 23 and Figure 24–The Figures contains sequence information of the flanking sequences associated with the HB4 wheat insert into the wheat genome
- Figure 25–Contains the sequence information of the junction sequences
- Appendix 1–Contains the long insert sequence, including the flanking sequences of the wheat genome
- Appendix 2–Contains the short insertion sequence, including the flanking sequences of the wheat genome
- Supplement Report A3c Molecular Characterisation.

Release of Information

Trigall Genetics is submitting the information in this application for review by Food Standards Australia New Zealand (FSANZ) for amendment to the Food **Standard 1.5.2** *Food Produced Using Gene Technology*. Trigall Genetics holds proprietary rights to the extent allowable by law to all such information and by submitting this information, Trigall Genetics does not authorise its release to any third party except to the extent it is duly requested under the *Freedom of Information Act 1982* (*FOI Act*) or in compliance with the responsibility of FSANZ to publish documents required under Sections 8, 8(A), 8(C) and 8(D) of the *FOI Act*; and this information is responsive to the specific aforementioned request. Accordingly, except as specifically stated above, Trigall Genetics does not authorise the release, publication, or other distribution of this information (including website posting or otherwise), nor does Trigall Genetics authorise any third party to use, obtain, or rely upon this information, directly or indirectly, as part of any other application or for any other use, without Trigall Genetics's prior notice and written consent. Submission of this information does not in any way waive Trigall Genetics's rights (including rights to exclusivity and compensation) to such information.

H. Other Confidential Information

No additional confidential material is included in this submission document.

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I. Exclusive Capturable Commercial Benefit

Trigall Genetics acknowledges that the proposed amendment to the Standard will likely result in an exclusive capturable commercial benefit being accrued to Trigall Genetics as defined in Section 8 of the *FSANZ Act*.

J. International and Other National Standards

The Argentina's National Advisory Commission on Agricultural Biotechnology (CONABIA) and the Biotechnology Directorate of the Ministry of Agroindustry completed their internal reviews on the agro-ecosystem safety assessment and concluded that wheat event IND-ØØ412-7 is as safe for the environment as conventional wheat. On April 26, 2016, the Argentina's National Service on Agricultural Food Health and Quality (Servicio Nacional de Sanidad y Calidad Agroalimentaria, SENASA) within the Argentina´s Ministry of Agroindustry completed the review on food/feed safety and concluded that wheat event IND-ØØ412-7 is as safe as conventional wheat from a food/feed safety perspective (SENASA, 2016). Recently, The Argentinian Ministry of Agriculture, Livestock and Fisheries, granted deregulated status to wheat IND-ØØ412-7 conditioning its commercialization to import approval from Brazil (Resolución 41/2020; MAGyP, 2020). Further steps to complete the approval process for commercialisation are currently underway and are summarised (Table 1).

Trigall Genetics joint venture partner Bioceres have participated in the development of a soybean variety also containing the *HaHB4* gene (soybean event IND-ØØ41Ø-5), which has also received several regulatory assessments (e.g., MAGyP, 2015; USDA,2019).

Responsible environmental stewardship and deployment of biotechnology-derived products are important to Trigall Genetics. The joint venture partner Bioceres uses INDEAR as its Research and Development Company. INDEAR is a member of Excellence Through Stewardship® (ETS), an industry-coordinated initiative that promotes the global adoption of stewardship programs and quality management systems for the full life cycle of biotechnology-derived plant products. The ETS "*Guide for Product Launch Stewardship of Biotechnology-Derived Products*" (ETS, 2013) also references and is consistent with the product launch policies of the Biotechnology Industry Organisation and Crop Life International.

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Table 1: Current Applications and Approval Status for IND-ØØ412-7

Country	Competent National Authority	Type of Authorisation	Approval Status
United States	United States Department of Agriculture (USDA)	Determination of non-regulated status	Under Evaluation (2021)
	Food and Drug Administration (FDA)	Food and Feed	Under Evaluation (2018)
Argentina[a]	Ministerio de Ganadería Agricultura y Pesca (MAGyP)	Food, Feed and Cultivation/Production	Approved (2020)
Brazil	Comissão Técnica Nacional de Biossegurança (CTNBio)	Food and Feed	Under Evaluation (2019)
Uruguay	Ministerio de Ganadería, Agricultura y Pesca (GNBio)	Food, Feed and Cultivation/Production	Under Evaluation (2015)
Paraguay	The National Commission of Agricultural and Forestry Biosafety (CONBIO)	Food and Feed	Submitted (2016)
		Cultivation/Production	Submitted (2018)
Colombia	Instituto Nacional de Vigilancia de Medicamentos y Alimentos (INVIMA)	Food	Under Evaluation (2019)
	Instituto Colombiano Agropecuario (ICA)	Feed	Submitted (2019)

[a] Bioceres obtained CONABIA (Environmental) and SENASA (Food and feed) approvals in 2016.

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K. Statutory Declaration – Australia

See attached statutory declaration provided separately.

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L. Checklists Provided With Application

General Requirements

General requirements (3.1.1)		
Check	**Page No.**	**Mandatory requirements**
☒	2	A Form of application ☒ Application in English ☒ Executive Summary (separated from main application electronically) ☒ Relevant sections of Part 3 clearly identified ☒ Pages sequentially numbered ☒ Electronic copy (searchable) ☒ All references provided
☒	3	B Applicant details
☒	3	C Purpose of the application
☒	3	D Justification for the application ☒ Regulatory impact information ☒ Impact on international trade
☒	4	E Information to support the application ☒ Data requirements
☒	5	F Assessment procedure ☒ General ☐ Major ☐ Minor ☐ High level health claim variation
☒	5	G Confidential commercial information ☒ CCI material separated from other application material ☒ Formal request including reasons ☒ Non-confidential summary provided
☒	5	H Other confidential information ☒ Confidential material separated from other application material ☒ Formal request including reasons
☒	6	I Exclusive Capturable Commercial Benefit ☒ Justification provided
☒	6	J International and other national standards ☒ International standards ☒ Other national standards
☒	8	K Statutory Declaration
☒	9	L Checklist/s provided with application ☒ 3.1.1 Checklist ☒ All page number references from application included ☒ Any other relevant checklists for Chapters 3.2–3.7

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Foods Produced Using Gene Technology

Foods produced using gene technology (3.5.1)		
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Part 2: Specific Data Requirements for Safety Assessment

The following information is provided to support an application for a new genetically modified food. The details presented are in accordance with Section 3.5.1. of the FSANZ Application Handbook as at, 1 July 2019.

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Supplement Report B5 Compositional analysis with glufosinate
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Abbreviations, Acronyms and Definitions[2]

Abbreviation	Definition
ABA	Abscisic Acid
ADF	Acid detergent fibre
ADP	Adenosine diphosphate
ANOVA	Analysis of Variance
ATP	Adenosine triphosphate
AUG	Start codon
Backbone DNA	DNA associated with construct backbone
bar	Gene from *Streptomyces hygroscopicus*
bp	Base pair
Cadenza	Parental variety for IND-ØØ412-7
CBI	Confidential Business Information
CDS	Coding sequence
Chr	Chromosome
CONABIA	Argentina National Advisory Commission on Agricultural Biotechnology
C-t	Carboxy terminal region
DArtT	Diversity Arrays Technology
DW	Dry weight
DIG	Digitonin
DNA insert	DNA sequence from *pIND4-HB4* or *pIND4-Bar* integrated into the wheat genome
dNTP	Deoxy nucleotide triphosphate
dsRNA	Double-stranded RNA
ELISA	Enzyme Linked Immunosorbent Assay
EPA	Environmental Protection Agency
ET	Ethylene
ETS	Excellence Through Stewardship
EU	European Union
FAO	Food and Agriculture Organization of the United Nations
FARRP	Food Allergy Resource Research Program and the University of Nebraska Lincoln
FDA	Food and Drug Administration
FW	Fresh weight
GE, GM, GMO	Genetically engineered/modified/modified organism
HaHB4	Transcription factor gene from sunflower (*Helianthus annuus*)
HAHB4	Protein encoded by the *HaHB4* gene
HD	Homeodomain
IND-ØØ412-7	OECD unique identifier for the wheat event selected for commercial approval
JA	Jasmonic acid
JP	Joining point
Kb	Kilobase
kDa	Kilodaltons
LC-MS	Liquid Chromatography Mass Spectrometry

[2] NOTE: Abbreviations of units of measure and of physical and chemical quantities are used according to the standard format described in Instructions to Authors in the Journal of Biological Chemistry (http://www.jbc.org/).

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LOD	Limit of detection
LOQ	Limit of quantification
LZ	Leucine zipper
MALDI-TOF	Matrix-Assisted Laser Desorption/Ionization - Time of flight
Mt	Metric tonnes
NDF	Neutral Detergent Fibre
NGS	Next generation sequencing
OECD	Organisation for Economic Cooperation and Development
OGTR	Office of the Gene Technology Regulator
ORF	Open reading frame
PacBio	Pacific Biosciences of California
PAT	Phosphinothricin-N-acetyl transferase
PCR	Polymerase chain reaction
RT-qPCR	Reverse transcription-qualitative polymerase chain reaction
SDAP	Structural Database of Allergenic Proteins
TF	Transcription factor
US	United States of America
WHO	World Health Organization
WT	Wild type

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A. Technical Information on the Food Produced Using Gene Technology

A.1. Nature and Identity of the Genetically Modified Food

A.1(a) A description of the GM organism from which the new GM food is derived. The description must include the nature and purpose of the genetic modification.

The wheat event IND-ØØ412-7 was developed by transforming the wheat variety Cadenza, with the plasmid vectors *pIND4-HB4* and *pIND4-Bar* using particle bombardment. The event was developed to confer increased tolerance to environmental stresses avoiding reduction of crop yield and exhibits tolerance to glufosinate-based herbicides (Table 2).

The plasmid *pIND4-HB4* contains a cassette for the expression of the transcription factor *HaHB4* that confers tolerance to environmental stress. The plasmid *pIND4-Bar* contains a cassette for the expression of the *bar* gene coding for the enzyme phosphinothricin N-acetyl transferase (PAT), providing herbicide tolerance.

Table 2: Summary of Genes, Intended Traits, and Benefits in HB4 Wheat

Construct	Gene Target	Mechanism	Intended Trait	Intended Benefit
pIND4-HB4	*HaHB4*: codes for a sunflower transcription factor belonging to the homeodomain-leucine zipper I subfamily[1]	*De novo* expression	Environmental stress tolerance	Yield protection under abiotic stress
pIND4-Bar	*Bar*: phosphinothricin N-acetyl transferase[2]	*De novo* expression	Tolerance to glufosinate-based herbicides	Provides post emergence herbicide tolerance for the on-farm management of weeds

[1] The HAHB4 protein has previously been evaluated in soybean by US FDA EFSE: early food safety assessment: NPC 00016 (FDA, 2015)
[2] The PAT protein has previously been evaluated by FSANZ in several crops. For example: Soybean (A481, A1046, A1073, A1081); Canola (A372, A1140); Maize (A375, A380, A385, A386, A446, A543, A1106, A1116, A1118, A1192); Cotton, A518, A533, A1028, A1040, A1080); Rice (A589).

Details of the identity of genes and expression products are provided in **Supplement Report A.1**.

A.1(b) The name, number or other identifier of each of the new lines or strains of GM organism from which the food is derived.

In accordance with OECD '*Guidance for the Designation of a Unique Identifier for Transgenic Plants*', the OECD Unique Identification Code for the wheat event is IND-ØØ412-7.

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A.1(c) The name the food will be marketed under (if known).

The wheat containing the environmental stress tolerance technology will be marketed as:

- HB4 Wheat

This wheat will be marketed under a variety of labels depending on the background wheat variety and licenced user of the event.

A.2. History of use of the host and donor organisms

A.2(a) For the donor organism(s) from which the genetic elements are derived:

A.2(a)(i) Any known pathogenicity, toxicity or allergenicity of relevance to the food

The donor organisms of all the genetic elements include in the constructions used to obtain IND-ØØ412-7 wheat have a history of use and/or exposure, as described in the next section.

Although some of the donor organisms may be related with pathogenicity (e.g. *A.tumefaciens* is a plant pathogen, some *E.coli* strains are pathogenic), none of genetic elements used to obtain wheat event IND-ØØ412-7 is associated to pathogenic properties.

No toxicity or has been reported for any of the donors and/or elements used to obtain HB4 wheat.

Concerning allergenicity, none of the donor organisms is recognised as a major allergen source and, even when allergenic components have been reported for some of them (i.e., sunflower), the genetic elements included in the constructions used to obtain HB4 wheat are not associated to them.

A.2(a)(ii) History of use of the organism in the food supply or history of human exposure to the organism through other than intended food use (e.g., as a normal contaminant)

Donor DNA of the inserts for event IND-ØØ412-7 consists of both coding and non-coding genetic elements from two plasmids *pIND4-HB4* and *pIND4-Bar* as described in Section A.3(b). The *pIND4-HB4* coding sequence is from sunflower (*Helianthus annuus*) and for *pIND4-Bar* from *Streptomyces hygroscopicus.* The non-coding elements of both plasmids are from maize (*Zea mays*) and *Agrobacterium tumefaciens*.

***Helianthus annus* L. (*HaHB4*- donor)**

The biology and history of sunflower has been widely reviewed (see for example CFIA 2015; Putnam et al., 2021). The development of the commercial sunflower has been a multi-national effort spanning continents and thousands of years. The sunflower is native to North America and was first grown as a crop by indigenous tribes over 4,500 years ago. Native Americans cultivated the sunflower from its original bushy, multi-headed type to produce a single-stemmed plant bearing a large flower.

The crop's multiple uses included milling for flour or meal production to make bread and cakes. Seeds were roasted, cracked and eaten whole, either as a snack or mixed with other grains, nuts and pulses into a type of granola.

The early Americans also discovered that sunflower oil could be extracted and used for cooking. Aside from the crop's value as a food, archaeologists have shown sunflower had a variety of non-food uses. The sunflower's oils and pigments were used as a sunscreen or the basis for a purple dye for skin, hair, or textile decoration, while the plant's sturdy, fibrous stem was exploited in construction.

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***Streptomyces hygroscopicus* (*Bar* donor)**

The *bar* gene in HB4 wheat is identical to that originally cloned from *Streptomyces hygroscopicus* (Murakami et al., 1986) and demonstrated to be useful as a selectable marker in other bacteria (Thompson et al., 1987) and in plants (Block et al., 1987; Takano and Dayan 2020).

Streptomyces hygroscopicus is a common saprophytic bacterial species that is found worldwide. Soil is the predominant habitat, but these organisms may also be isolated from water.

Streptomyces hygroscopicus produces a variety of useful antimicrobial and herbicidal compounds (Dunne et al., 1998), of which the PAT enzyme confers phosphinothricin tripeptide (phosphinothricin or bialaphos) tolerance. This tolerance is conferred through inactivation by transfer of an acetyl group. Acetyltransferase activity has been identified in six other bacterial species from five different genera of common soil bacteria. This is thought to have evolved as a protective mechanism to protect these microorganisms from antimicrobials produced by both themselves and other competing microorganisms. Consequently, natural resistance to phosphinothricin and N-acetyltransferase has also been reported in various genera of soil bacteria (Bartsch and Tebbe, 1989).

Recently, numerous works report their important symbiotic relationships with plants and animals (Kaltenpoth et al., 2005; Behie et al., 2016). A recent work describes the first clearly documented case of their mutualism with vertebrates, sea turtles (Sarmiento-Ramirez et al., 2014). In almost all reported cases the streptomycetes protect the host or its food resources from pathogenic fungi.

Streptomyces species very rarely cause human disease but can be detected as common colonisers of human bodies, especially the skin, the respiratory tract, the guts, and the genital tract using molecular techniques (Herbrik et al., 2020). In general, streptomycetes cause suppurative granulomatous tissue changes (Dunne et al., 1998; Herbrik et al., 2020). However, their clinical manifestations and isolations are rare. It is expected that humans would be exposed to these microorganisms and anti-microbial compounds directly through the consumption of roots and other vegetables that are eaten fresh.

The PAT protein is expressed by several transgenic crops that have been in commercial production for many years. FSANZ have not identify any public health or safety concerns associated with the expression of PAT, as encoded by the *pat* or *bar* gene, in numerous assessments (for example, Soybean (A481, A1046, A1073, A1081); Canola (A372, A1140); Maize (A375, A380, A385, A386, A446, A543, A1106, A1116, A1118, A1192); Cotton, A518, A533, A1028, A1040, A1080); Rice (A589). Therefore, this protein has been well characterised and demonstrated to be non-toxic to humans and animals.

Non-coding sequences

The promoter and terminator sequences used in HB4 wheat are derived from common plants or plant pathogens. These genetic elements constitute a minute component of their respective genomes, no genetic elements associated with pathogenicity have been used in the construction of HB4 wheat.

Many of the organisms from which these elements are derived are model species in plant science with a history of safe use.

Expression of the *HaHB4* and *bar* genes are driven by the Ubiquitin promoter from *Zea mays*, a crop plant that has the greatest global production of any crop species and has a long history of safe use for food, animal feed and industrial products.

Poly (A) signals for the termination of *HaHB4* and *bar* gene transcription are derived from *Agrobacterium tumefaciens* is a soil born, gram-negative bacterium that has been extensively studied since it was identified as the causative agent of crown gall disease in plants (Depicker et al., 1982).

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A.2(b) A description of the host organism into which the genes were transferred:

A.2(b)(i) Its history of safe use for food

Wheat is the world's second largest food crop, following rice. It has a long history in the diets of humans across the entire world. Wheat is the major winter crop grown in Australia with production predominantly in Western Australia, New South Wales, South Australia, Victoria and Queensland (31 million MT p/a; ABARES 2020).

Most Australian wheat is sold overseas with Western Australia the largest exporting state. The major export markets are in the Asian and Middle East regions and include Indonesia, Japan, South Korea, Malaysia, Vietnam and Sudan.

Similarly, in New Zealand, wheat is a winter crop with production almost exclusively in the South Island (approximately 400 000 MT p/a; StatsNZ 2020[3]). Wheat grown in New Zealand is primarily used domestically for food and feed for livestock.

The biology of wheat is fully described in several OECD documents (OECD, 1999a, 2003, 2006) and other regulatory publications (CFIA, 2012; OGTR, 2021).

Details of the pathogenicity, toxicity or allergenicity of wheat are described in the OECD Consensus Document on Compositional Considerations for New Varieties of bread wheat: Key Food and Feed Nutrients, Anti-nutrients and Toxicants (OECD 2003).

Whole grain wheat is a major source of nutrients such as protein, B vitamins and minerals for humans and livestock. Globally, wheat is the largest food crop in terms of area allocation (nearly 25% of global arable land) and is the second most produced cereal crop after maize (Velu and Singh, 2013). Wheat accounts for 56% of the global coarse grain production and is the staple food for more than one third of the world's population providing more calories and protein in the human diet than any other crop (Curtis, 2002; IDRC, 2010).

Wheat is among the 'Big Eight' group of foods that account for ~90% of all food allergies in the U.S. (FARRP, 2014). Wheat is one of the most common allergenic foods associated with IgE-mediated reactions in the world but has only rarely been reported to cause anaphylaxis (Takizawa et al 2001). Wheat is associated with the (IgE-mediated) conditions known as baker's asthma, resulting from the inhalation of wheat flour, and atopic dermatitis. The ingestion of wheat flour has also produced anaphylaxis in rare instances in children.

Wheat, along with other gluten-containing cereals such as rye and barley, is associated with non-IgE reactions such as coeliac disease, a condition of gluten-sensitive enteropathy, which affects genetically predisposed individuals (FAO 2001). The response is triggered by gliadin (Howdle and Blair 1992) and is considered an autoimmune disease where the lining of the small intestine is damaged by gluten (Caio et al., 2020).

Currently, the only treatment for coeliac disease is a life-long, strict gluten-free diet leading to improvement in quality of life, ameliorating symptoms, and preventing the occurrence of refractory coeliac disease, ulcerative jejunoileitis, and small intestinal adenocarcinoma and lymphoma.

No sequences associated with known toxins or allergens were used in creating the wheat event proposed in this application.

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[3] Agricultural production statistics: June 2020 (provisional). Retrieved March 2020, from: https://www.stats.govt.nz/information-releases/agricultural-production-statistics-june-2020-provisional

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ORDER:	*Poales*
FAMILY:	*Poaceae*
GENUS:	*Triticum*
SPECIES:	*T. aestivum L*
COMMON NAME:	Wheat, Bread Wheat

A.2(b)(ii) The part of the organism typically used as food

Wheat grain is the primary part consumed as food and as animal feed. Vegetative material may also be directly grazed by livestock or harvested for hay. Young seedlings may also be added to drinks such as smoothies.

A.2(b)(iii) The types of products likely to include the food or food ingredient

In Australia and New Zealand, consumer research has confirmed that the major uses of bread wheat involve some level of processing/milling. The versatility of bread wheat has led to a range of uses, with the majority being centred around the grain. Bread wheat is typically ground to a flour that is then used for the manufacture of bread and other baked products. However, whole grain is also used to supplement animal feed.

Other than its primary use as a human food source, wheat has several alternative uses. These include, but are not limited to, use in animal feed, conversion of wheat starch to ethanol, brewing of beer, wheat based raw materials for cosmetics, wheat protein in meat substitutes and to make wheat straw composites (OGTR, 2021).

A.2(b)(iv) Whether special processing is required to render food derived from the organism safe to eat

Although bread wheat products are typically processed, the grain does not require any special processing for it to be safe for consumption. It is noted, however, that up to 1% of the population may be affected by an autoimmune disease associated with exposure to gluten (i.e., coeliac disease; Caio et al., 2020).

A.3. The nature of the genetic modification

A.3(a) A description of the method used to transform the host organism

The wheat event IND-ØØ412-7 was developed by transforming the variety Cadenza with the plasmids *pIND4-HB4* and *pIND4-Bar* to produce the proteins HAHB4 and PAT. These plasmids are described in detail in Section A3(b) with the PAT protein assessed previously by FSANZ in numerous applications (see Table 2). The transformation protocol is described in Figure 1.

Conclusion of the Development of HB4 Wheat

Wheat event IND-ØØ412-7 was developed by co-transforming the bread wheat variety Cadenza with *pIND4-HB4* and *pIND4-Bar*. Transformation introduced DNA sequences (*HaHB4* and *bar*) intended to provide tolerance to environmental stresses and tolerance to herbicides containing glufosinate.

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All genetic elements used to create HB4 wheat were derived from the genomes of organisms present in the natural environment. Wheat and Soybean events containing the coding sequences of *HaHB4* and *bar* have been assessed and approved by other regulatory agencies and GM events from a range of food crops containing the coding sequence of the *bar* gene have been assessed and approved by FSANZ from numerous independent submissions.

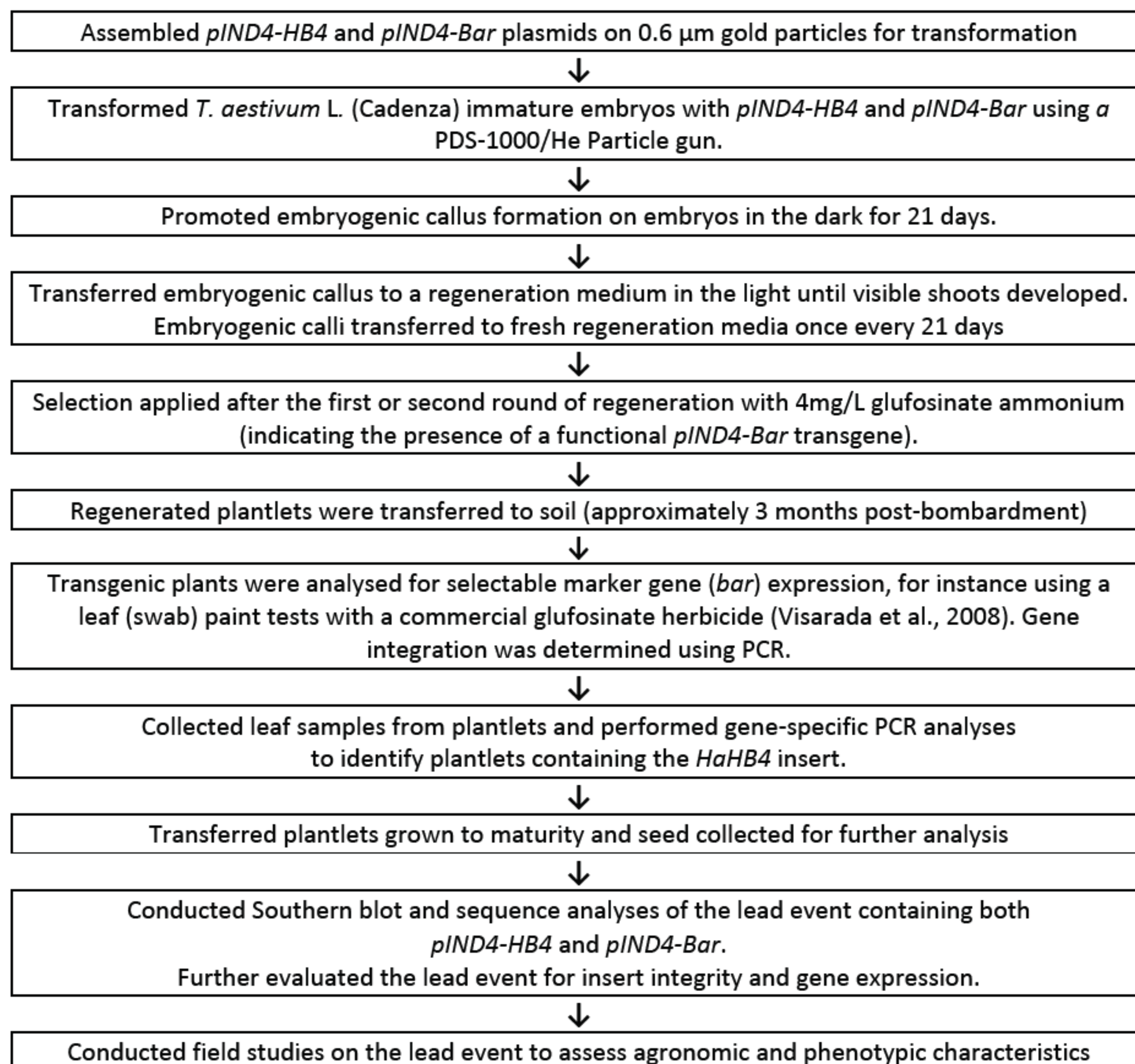

Figure 1. The Development and Selection of HB4 Wheat Transformed with *pIND4-HB4* and *pIND4-Bar*

The protocol for particle bombardment transformation of wheat was adapted from published literature (Barcelo and Lazzeri, 1995; Pastori et al., 2001; Rasco-Gaunt et al., 2001; Sparks and Jones, 2014)

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A.3(b) A description of the construct and the transformation vectors used

HB4 wheat was developed by co-transforming the variety Cardenza with *pIND4-HB4* and *pIND4-Bar*. All genetic elements were derived from the genomes of species commonly found in the environment and/or the food chain. The resulting wheat variety possess tolerance to environmental stress and tolerance to herbicides containing glufosinate.

The *pIND4-HB4* and *pIND4-Bar* plasmids were constructed using the same parental plasmid and therefore share identical backbone sequences. Plasmids *pIND4-HB4* and *pIND4-Bar* are based on a set of plasmids that have been constructed on *pUC8* or *pUC19* plasmid sequences by fusing the same 1992 bp *PstI* fragment from the ubiquitin maize *Ubi-1* gene sequence (Christensen et al., 1992; Christensen and Quail, 1996) to the relevant genes. This *Ubi-1 PstI* fragment contains 899 bp of promoter sequence, 83 bp of 5' untranslated exon, and 1010 bp of first intron sequence upstream of restriction sites used in the construction of the chimeric genes (the promoter 'construct' is therefore *prUbi-1/Ubi-1Exon/Ubi-1Intron*). This *Ubi-1* promoter construct has been shown to be highly active in monocotyledonous plants (Christensen et al., 1992), to facilitate efficient transformation and to drive gene expression. The original intron present in the 5'-untranslated region of the *Ubi-1* gene (Christensen et al., 1992) was retained in all the constructs of this series because previous studies have shown that introns frequently exert a strong enhancing effect on transgene expression in cereals (Vasil et al., 1993).

The backbone contains a well-characterised bacterial origin of replication from pBR322, which enables maintenance of the plasmid in *Escherichia coli* and the ampicillin resistance gene (*Bla*) that functions as a selectable marker for maintenance in bacteria.

Maps of *pIND4-HB4* and *pIND4-Bar*, are provided in Figure 2 and Figure 3, respectively, with corresponding descriptions of the genetic elements provided in Table 3 and Table 4.

pIND4-HB4 is a 5,473 bp plasmid vector carrying the genetic elements to deliver the expression of the HAHB4 transcription factor in wheat. The plasmid *pIND4-HB4* contains a single gene cassette consisting of the Maize Ubiquitin promoter, the *HaHB4* coding sequence and the nopaline synthase Poly (A) signal for the termination of transcription of *HaHB4* (Figure 2).

pIND4-Bar is a 5,496 bp plasmid vector used during the co-transformation process. A plasmid map for *pIND4-Bar* is provided in Figure 3. As mentioned above, the plasmids were constructed using the same parental plasmid and therefore share identical backbone sequences (see Table 3 and Table 4).

The plasmid *pIND4-Bar* contains a single gene cassette consisting of the maize Ubiquitin promoter, the *bar* gene and the nopaline synthase Poly (A) signal for the termination of transcription of the *bar* gene (Figure 3).

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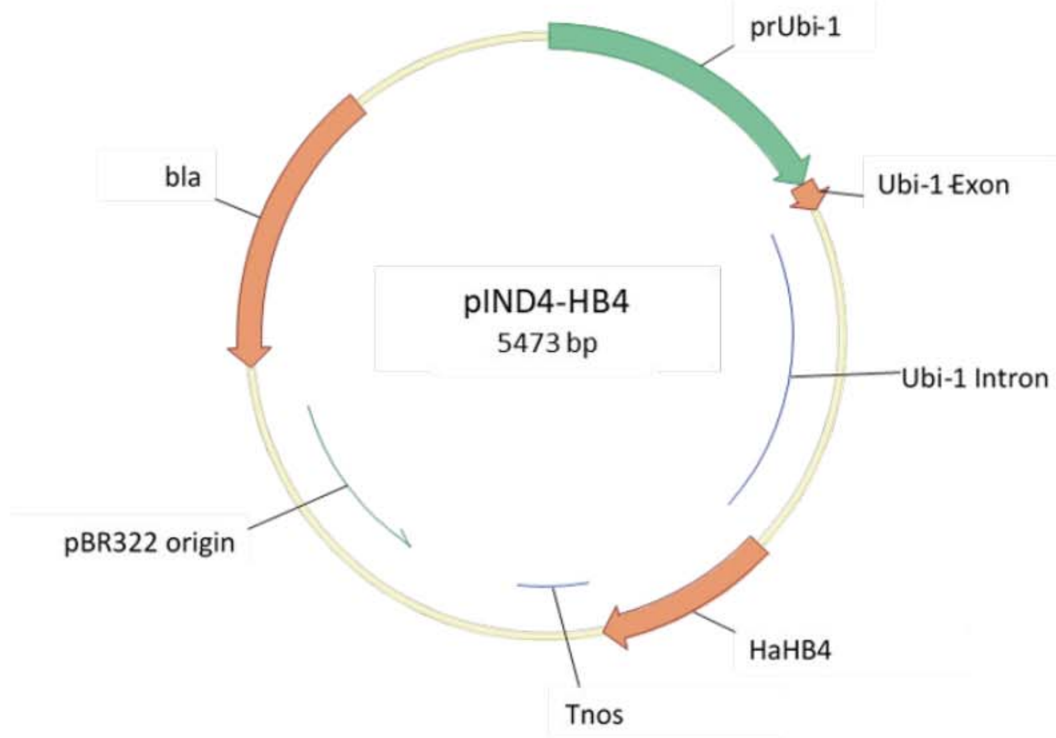

Figure 2. Plasmid Map of *pIND4-HB4*

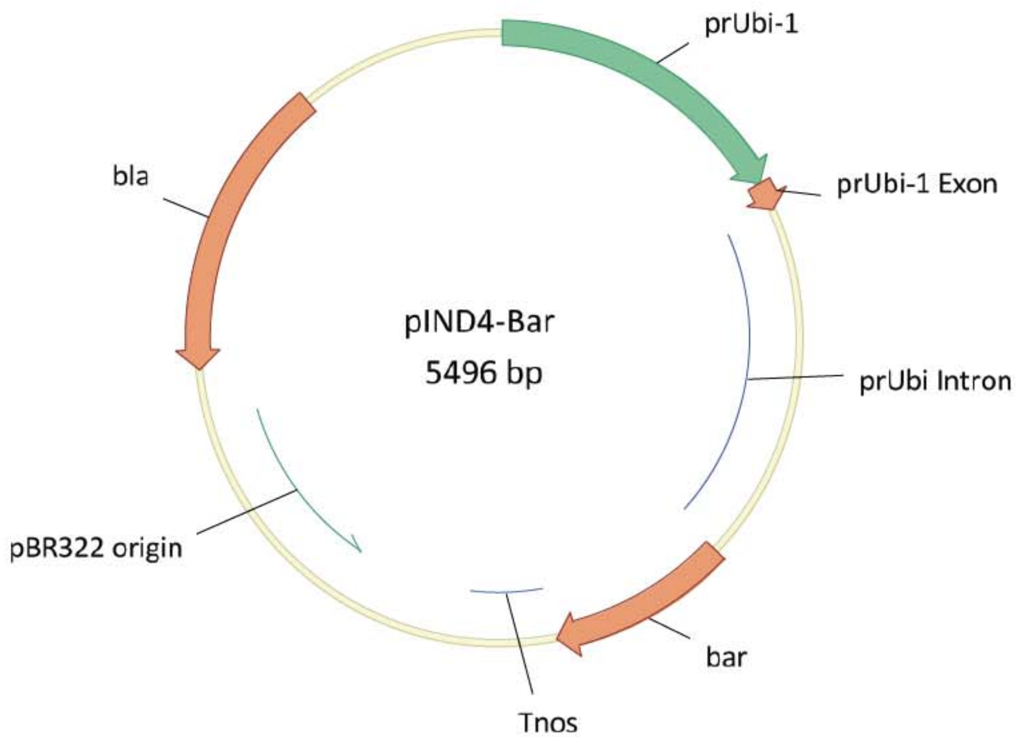

Figure 3.Plasmid Map of *pIND4-Bar*

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Table 3. Genetic Elements of *pIND4-HB4*

Genetic Element	*Origin*	Accession Number	Position (*pIND4-HB4*)	Size (bp)	Intended Function
1. Ubiquitin (Ubi-1) Promoter	*Zea mays*	S94464.1	1–898	898	*De novo* expression of the *HaHB4* gene (Christensen et al., 1992; Christensen and Quail, 1996)
2. Ubiquitin (Ubi-1) Exon	*Zea mays*	S94464.1	899–981	83	Ubi-1 gene 5' untranslated exon (Christensen et al., 1992; Christensen and Quail, 1996)
3. Intervening Sequence	*pUC8/pUC18*	L08959.1 and L08752.1	982–995	14	Sequence used for DNA cloning (Norrander et al., 1983)
4. Ubiquitin (Ubi-1) Intron	*Zea mays*	S94464.1	996–2,005	1,010	First intron of the Ubi-1 gene (Christensen et al., 1992; Christensen and Quail, 1996)
5. Intervening Sequence	*pUC8/pUC18*	L08959.1 and L08752.1	2,006–2031	26	Sequence used for DNA cloning
6. *HaHB4* coding sequence	*Helianthus annus*	AF339748.1	2,032–2,565	534	Generates mRNA that leads to HAHB4 providing environmental stress tolerance (Chan and Gonzalez 1994; Gago et al., 2002; Dezar et al., 2005a; Manavella et al., 2006)
7. Intervening Sequence	*pUC8/pUC18*	L08959.1 and L08752.1	2,566–2,581	16	Sequence used for DNA cloning
8. NOS-ter; poly(A)signal of nopaline synthase gene	*Agrobacterium tumefaciens*	V00087.1	2,582–2,834	253	Poly (A) signal for the termination of *HaHB4* transcription (Depicker et al., 1982)
9. Intervening Sequence	*pUC8/pUC18*	L08959.1 and L08752.1	2,835–3,239	405	Sequence used for DNA cloning
10. pBR322 origin of replication	*Synthetic* pBR322 plasmid	J01749.1	3,240–3,859	620	Plasmid origin of replication (Yanisch-Perron et al., 1985)
11. Intervening Sequence	*pUC8/pUC18*	L08959.1 and L08752.1	3,860–4,013	154	Sequence used for DNA cloning
12. *Bla* coding sequence	*Escherichia coli*	AAB59737.1	4,014–4,874	861	β-lactamase gene encoding for ampicillin resistance allowing for selection of plasmid carrying bacteria (Olesen et al., 2004)
13. Intervening Sequence	*pUC8/pUC18*	L08959.1 and L08752.1	4,875–5473	599	Sequence used for DNA cloning

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Table 4. Genetic Elements of *pIND4-Bar*

Genetic Element	*Origin*	Accession Number	Position (*pIND4-Bar)*	Size (bp)	Intended Function
1. Ubiquitin (Ubi-1) Promoter	*Zea mays*	S94464.1	1–898	898	*De novo* expression of the *bar* gene (Christensen et al., 1992; Christensen and Quail, 1996)
2. Ubiquitin (Ubi-1) Exon	*Zea mays*	S94464.1	899–981	83	Ubi-1 gene 5' untranslated exon (Christensen et al., 1992; Christensen and Quail, 1996)
3. Intervening Sequence	*pUC8/pUC18*	L08959.1 and L08752.1	982–995	14	Sequence used for DNA cloning (Norrander et al., 1983)
4. Ubiquitin (Ubi-1) Intron	*Zea mays*	S94464.1	996–2,005	1,010	First intron of the Ubi-1 gene (Christensen et al., 1992; Christensen and Quail, 1996)
5. Intervening Sequence	*pUC8/pUC18*	L08959.1 and L08752.1	2,006–2041	36	Sequence used for DNA cloning
6. *bar* coding sequence	*Streptomyces hygroscpicus*	P16426.1	2,042–2,590	549	Generates mRNA that leads to phosphinothricin acetyltransferase (PAT) providing herbicide tolerance (Thompson et al., 1987; White et al., 1990)
7. Intervening Sequence	*pUC8/pUC18*	L08959.1 and L08752.1	2,591–2,607	17	Sequence used for DNA cloning
8. NOS-ter; poly(A) signal of nopaline synthase gene	*Agrobacterium tumefaciens*	V00087.1	2,608–2,860	253	Poly (A) signal for the termination of transcription of the *bar* gene (Depicker et al., 1982)
9. Intervening Sequence	*pUC8/pUC18*	L08959.1 and L08752.1	2,861–3,263	403	Sequence used for DNA cloning
10. pBR322 origin of replication	*Synthetic* pBR322 plasmid	J01749.1 and AF234297	3,264–3,883	620	Plasmid origin of replication (Yanisch-Perron et al., 1985)
11. Intervening Sequence	*pUC8/pUC18*	L08959.1 and L08752.1	3,884–4,037	154	Sequence used for DNA cloning
12. *Bla* coding sequence	*Escherichia coli*	AAB59737.1	4,038–4,898	861	β-lactamase gene encoding for ampicillin resistance allowing for selection of plasmid carrying bacteria (Olesen et al., 2004)
13. Intervening Sequence	*pUC8/pUC18*	L08959.1 and L08752.1	4,899–5496	599	Sequence used for DNA cloning

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A.3(c) A full molecular characterisation of the genetic modification in the new organism

This Section provides information that addresses the requirements for Part A.3(c) A full molecular characterisation of the genetic modification in the new organism, including:

(i) Identification of all transferred genetic material and whether it has undergone any rearrangements

(ii) A determination of the number of insertion sites, and the number of copies at each insertion site

(iii) Full DNA sequence of each insertion site, including junction regions with the host DNA

(iv) A map depicting the organisation of the inserted genetic material at each insertion site; and

(v) Details of an analysis of the insert and junction regions for the occurrence of any open reading frames (ORFs).

Further information is provided in the **Supplement Report A3c Molecular characterisation.**

A.3(c)(i) to (iii) Structure of the Insertion in HB4 Wheat

Wheat event IND-ØØ412-7 was generated by co-transformation of wheat explants with the plasmids *pIND4-HB4* and *pIND4-Bar* using a particle bombardment method. Molecular characterisation of the event shows a complex integration structure as is often the case in the use of particle bombardment (Altpeter et al., 2005). Due to this complex structure, a combination of Southern blot hybridisation and DNA sequencing analysis was used to characterise the event.

1. Southern blot hybridisation

The conventional approach to determine the copy number and integration patterns of transgenic events is to use Southern blot hydridisation. Genomic DNA of homozygous event IND-ØØ412-7 was digested with the restriction endonucleases *Hind*III, *Bam*HI and *Ase*I (Figure 4).

Assuming the occurrence of a single intact copy of each of the relevant sequences (i.e., from the start of *prUbi-1* to the end of *Tnos),* the minimum predicted sizes of the bands detected in each digest with the *HaHB4* (200 bp) and *bar* (282 bp) probes are summarised in Table 5.

There is one *Hind*III site in the pIND4 plasmids (*pIND4-HB4* or *pIND4-Bar*), located very close to 5´ pr*Ubi*-1 extreme. No other *Hind*III site was present in the plasmids (Figure 4). Assuming the occurrence of single, intact inserts containing the sequence of interest in the genome of IND-ØØ412-7, the minimum fragment size of hybridisation of the *HaHB4* probe would be 2,842 bp and that of the *bar* probe would be 2,860 bp.

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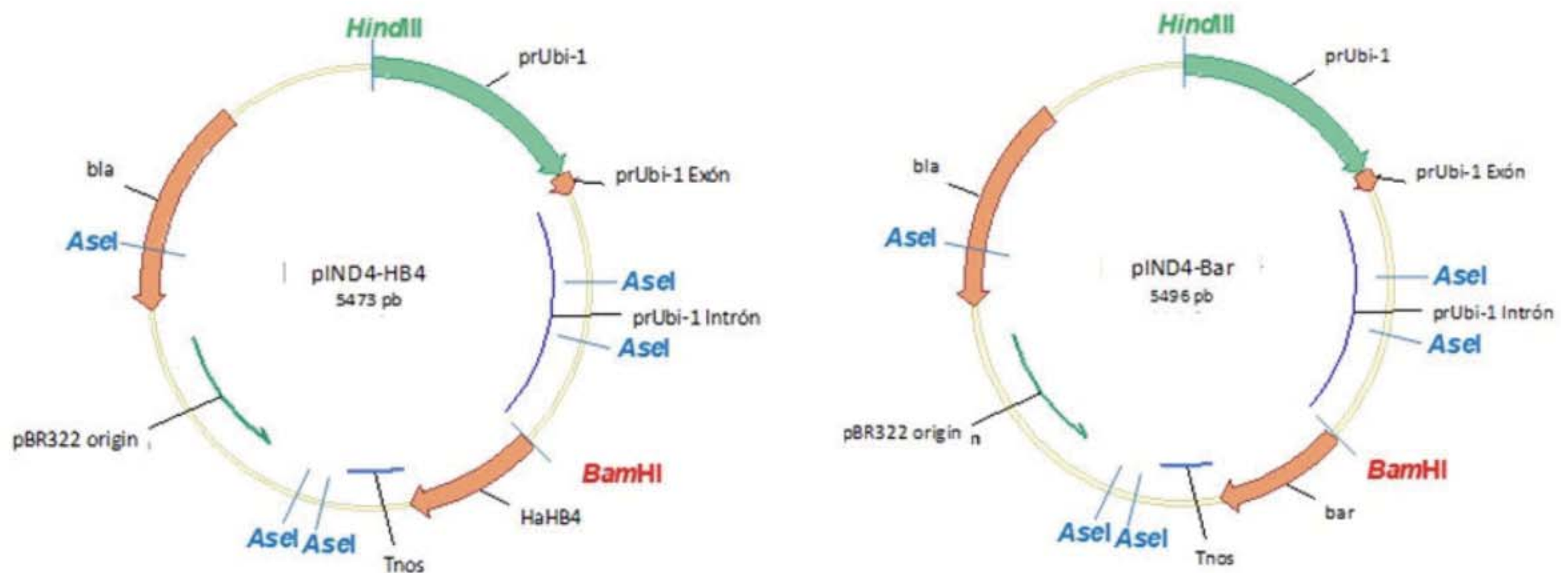

Figure 4. Restriction map of plasmids *pIND4-HB4* and *pIND4-Bar* inserted into Wheat Event IND-ØØ412-7.
The plasmids containing the CDSs of *HaHB4* (left) and *bar* (right) with their regulatory elements. *prUbi-1*: maize *Ubi-1* gene promoter region. *prUbi-1 Exon*: maize *Ubi-1* gene first exon. *prUbi-1* Intron: maize *Ubi-1* gene first intron. *HaHB4*: CDS of *HaHB4*. Bar: CDS of *bar*. Tnos: Poly(A) terminator sequence of the *nos* gene. pBR322 origin: *ColE1* replication origin. bla: *bla* gene. Maps show the position of restriction sites used for Southern blot hybridisation.

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Additionally, there is one *Bam*HI site in the pIND4 plasmid, located very close to the initiation codon of *HaHB4* or *bar* CDSs. No other *Bam*HI sites are present in the plasmids (Figure 4). Again, assuming the occurrence of single, intact inserts containing the sequence of interest in the genome of IND-ØØ412-7, the minimum fragment size of hybridisation of the *HaHB4* probe would be 825 bp and that of the *bar* probe would be 843 bp.

Finally, there are five *Ase*I restriction sites in both plasmids (Figure 4), two within of the sequence of interest, in the *Ubi*-1 intron, and three within the vector backbone, two closes to the end of *Tnos* and the other inside of *bla* gene. Assuming a single intact insertion, complete *Ase*I digestion in the insert should release a DNA segment of 1,313 bp long that contains the binding target for the *HaHB4* probe. Analogously, the *bar* probe will detect a DNA fragment of 1,331 bp.

Table 5. Predicted band sizes from Southern blot hybridisation

Restriction enzyme	Minimum fragment size predicted (bp)	
	***HaHB4* probe**	***bar* probe**
*Hind*III	2,842	2,860
*Bam*HI	825	843
*Ase*I	1,313	1,331

Minimum band sizes based on hybridisation with the *HaHB4* (200bp) and *bar* (282bp) probes

The analysis of hybridisation bands obtained with *HaHB4* probe allowed the assumption of the presence of three copies of in wheat event IND-ØØ412-7 (Figure 5). This was consistent from both *Hind*III and *Bam*HI digests. On the other hand, the analysis of hybridisation bands obtained with *bar* probe identified four bands with *Hind*III and suggested the presence of up to seven copies with *Bam*HI (Figure 6). However, when considering *Hind*III and *Ase*I the number of fragments is lower, indicating possible internal DNA rearrangements (Figure 6).

In brief, southern blots results showed a complex insertion pattern with multiple copies of each of the *HaB4* and *bar* genes with possible internal DNA rearrangements. Due to this complex arrangement a genomic sequencing approach was undertaken to fully resolve the insertion structure of HB4 wheat.

The detailed identification of the sequences in both inserts allowed the *ex-post* interpretation of the Southern blot hybridisation results, in terms of the origins and lengths of the bands detected. A schematic representation of the results of this analysis is given in Figure 7, Figure 8, Figure 9 and Figure 10.

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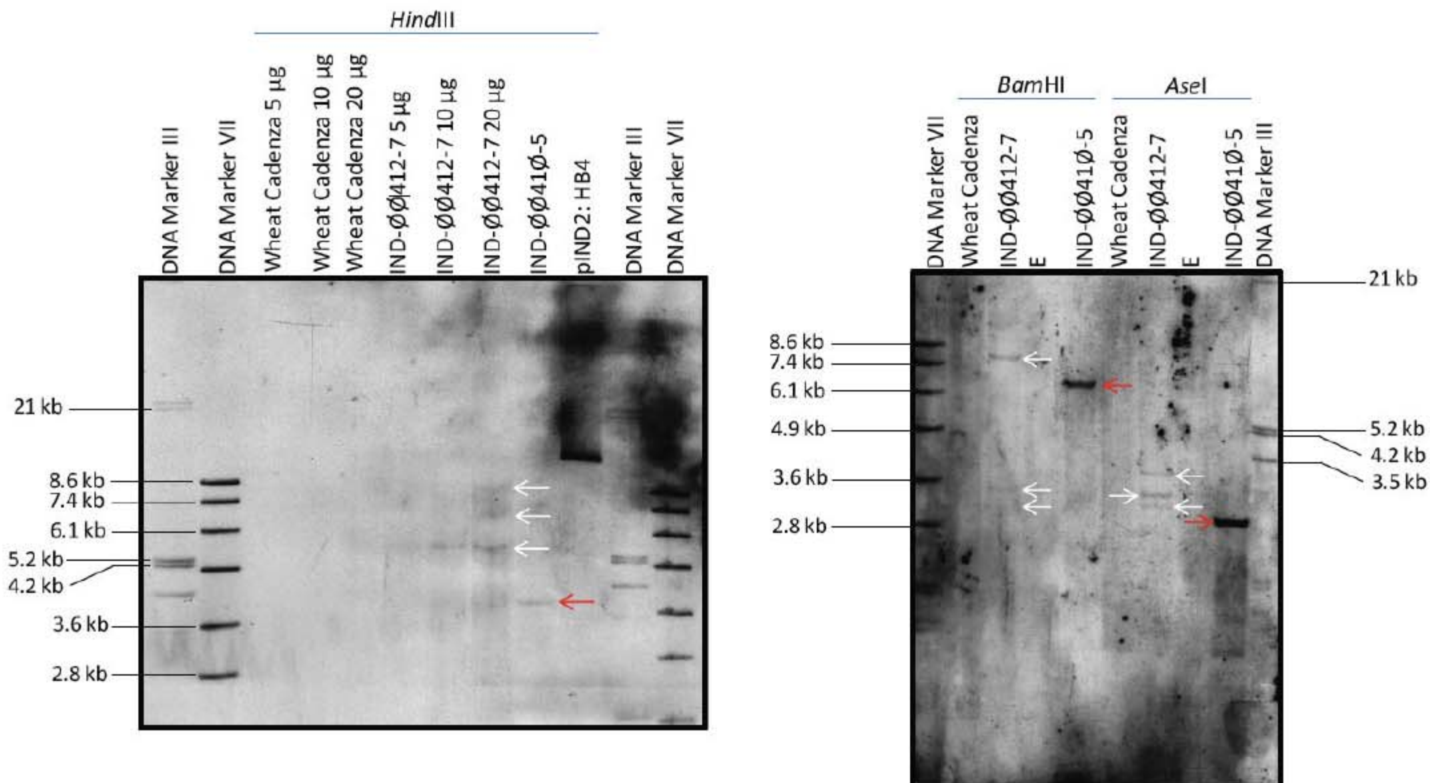

Figure 5. Southern Blots of IND-ØØ412-7 Plant DNA Digested with *Hind*III, *Bam*HI and *Ase*I and hybridised with DIG-labelled probes for *HaHB4* detection. DNA bands in IND-ØØ412-7 digests hybridising to probes are pointed with white arrows. HB4 Soybean transgenic plant (IND-ØØ41Ø-5) was used as a positive control and the hybridising band is indicated by a red arrow. DIG-labelled Marker VII and III ladder band sizes are indicated on the left and right, respectively, of the blots in kb. E: empty lane (no sample loaded).

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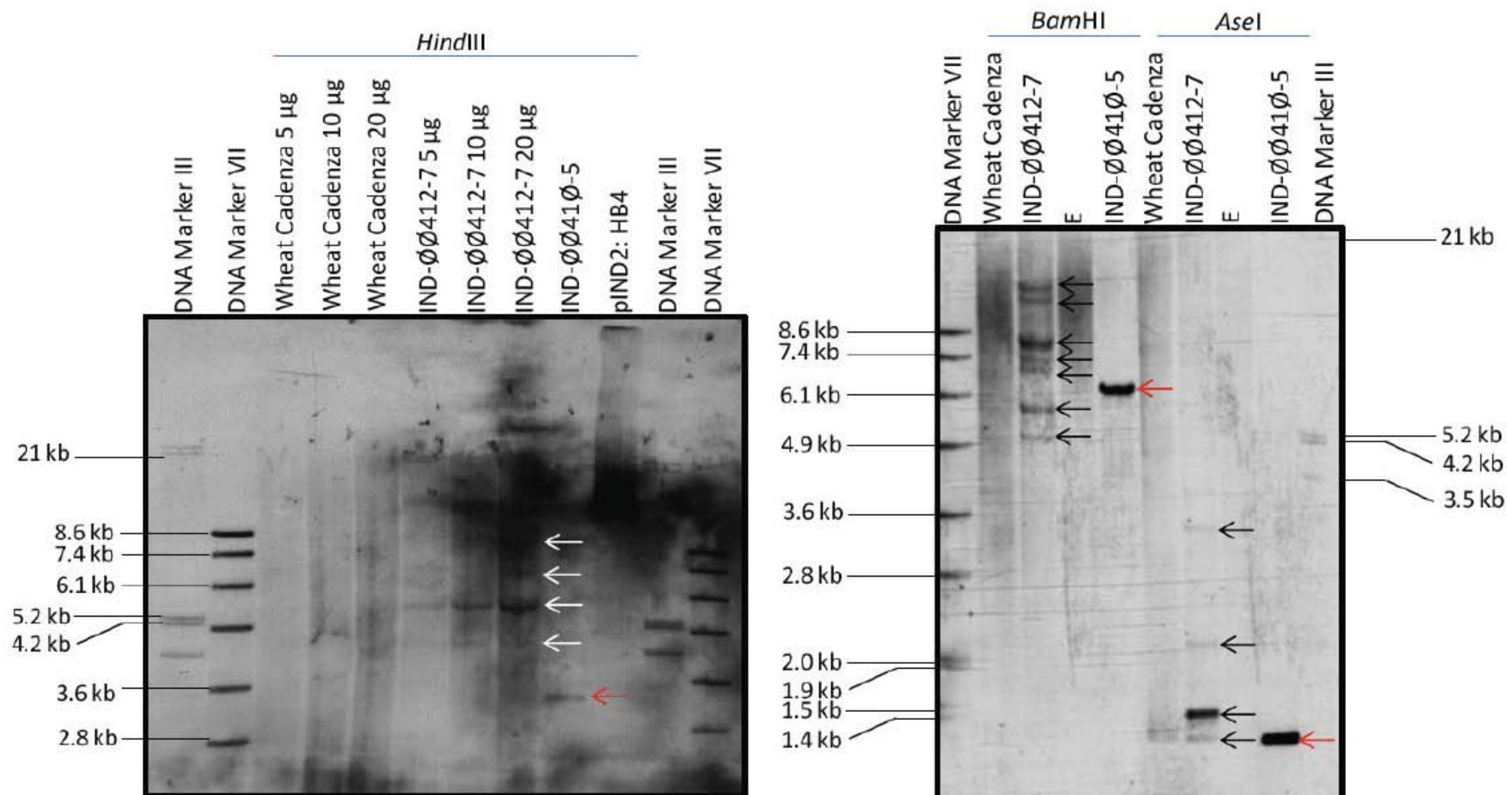

Figure 6. Southern Blots of IND-ØØ412-7 Plant DNA Digested with *Hind*III, *Bam*HI and *Ase*I hybridised with DIG-labelled probes for *bar* detection.
DNA bands in IND-ØØ412-7 digests hybridising to probes are pointed with white arrows. HB4 Soybean transgenic plant (IND-ØØ41Ø-5) was used as positive and hybridising band is pointed with red arrow. DIG-labelled Marker VII and III ladder band sizes are indicated on the left and right, respectively, of the blots in kb. E: empty lane (no sample loaded).

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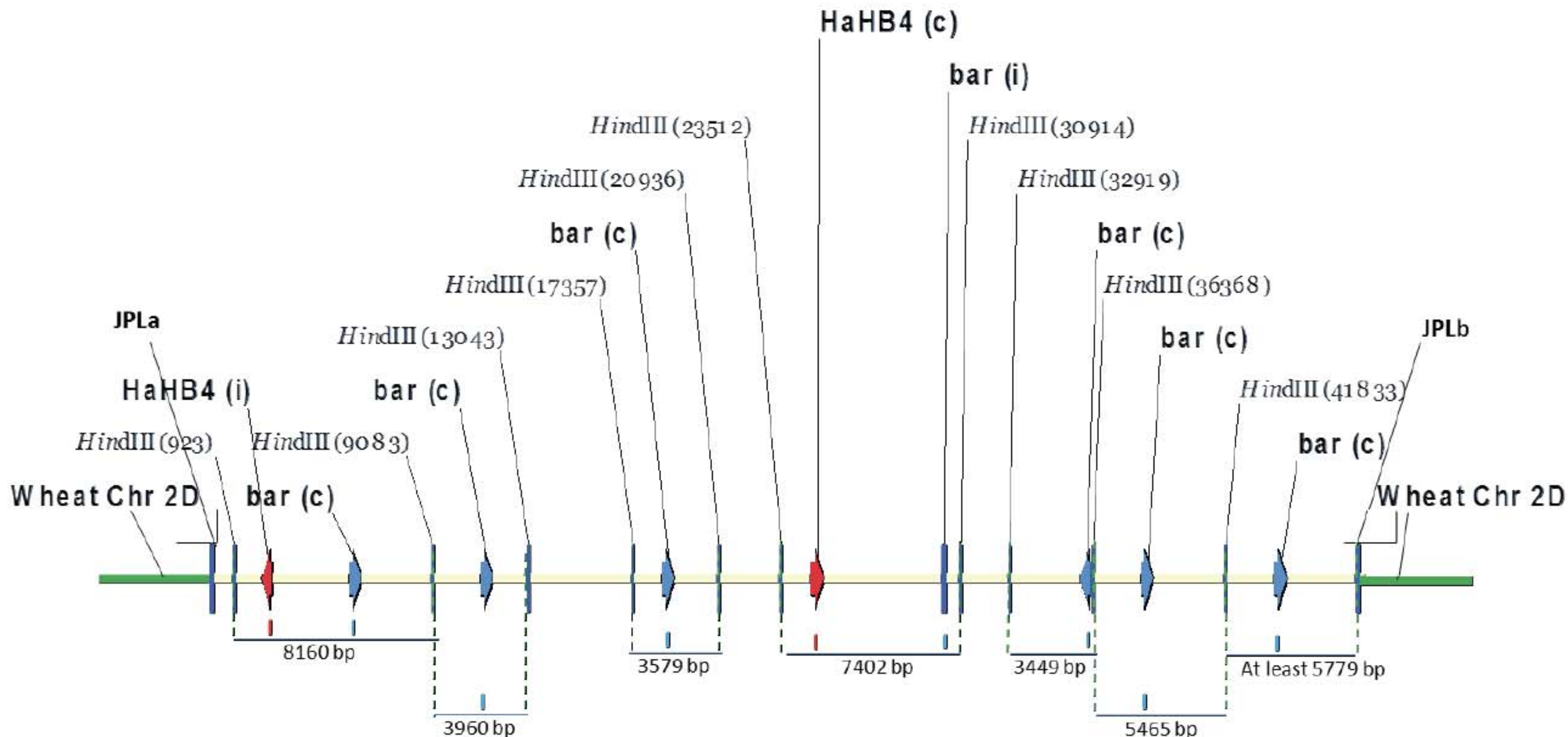

Figure 7. Schematic Representation of Fragments from the Long Insert Detected in the Southern Blot Hybridisation by DNA Digestion with *Hind*III.
Solid arrows indicate the locations of the *HaHB4* (red) and *bar* (blue) CDSs. The thick green line indicates the wheat genome. Below the insert, short vertical-coloured bars indicate the hybridisation sites for *HaHB4* (red) and *bar* (light blue) probes. Detected fragments are shown by thin horizontal blue lines with their size indicated underneath.

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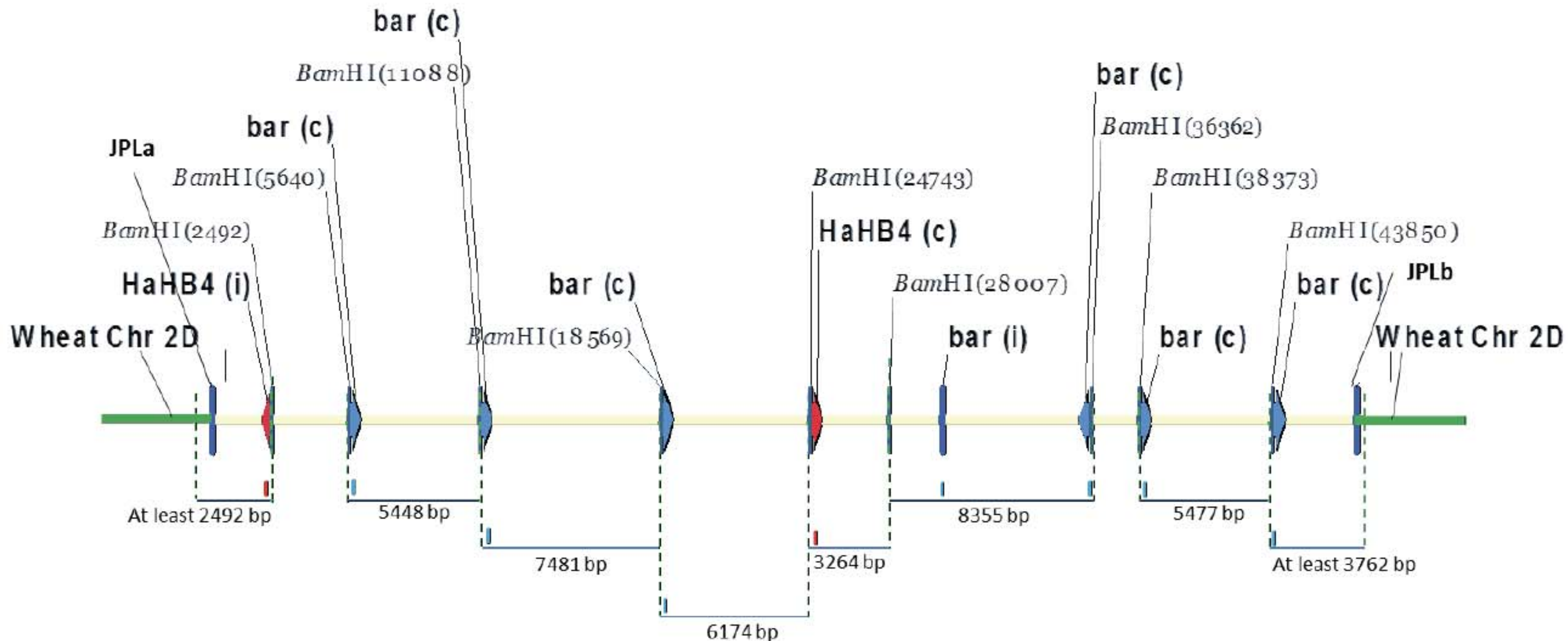

Figure 8. Schematic Representation of Bands from the Long Insert Detected in the Southern Blot Hybridisation by Digestion with *Bam*HI.
Solid arrows indicate the locations of the *HaHB4* (red) and *bar* (blue) CDSs. The thick green line indicates the wheat genome. Below the insert, short vertical-coloured bars indicate the hybridisation sites for *HaHB4* (red) and *bar* (light blue) probes. Detected fragments are shown by thin horizontal blue lines with their size indicated underneath.

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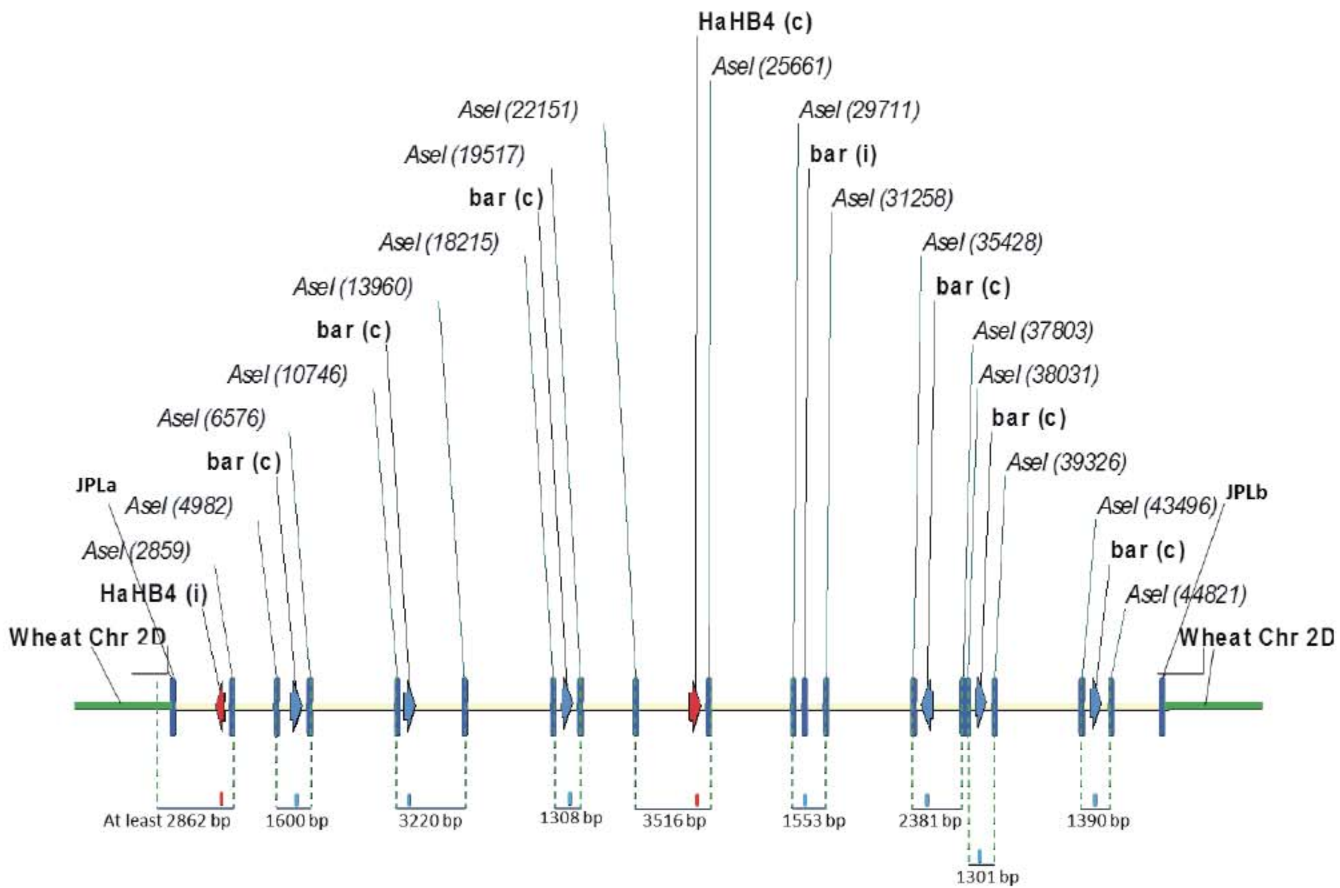

Figure 9. Schematic Representation of Bands from the Long Insert Detected in the Southern Blot Hybridisation by Digestion with *Ase*I.
Solid arrows indicate the locations of the *HaHB4* (red) and *bar* (blue) CDSs. The thick green line indicates the wheat genome. Below the insert, short vertical-coloured bars indicate the hybridisation sites for *HaHB4* (red) and *bar* (light blue) probes. Detected fragments are shown by thin horizontal blue lines with their size indicated underneath.

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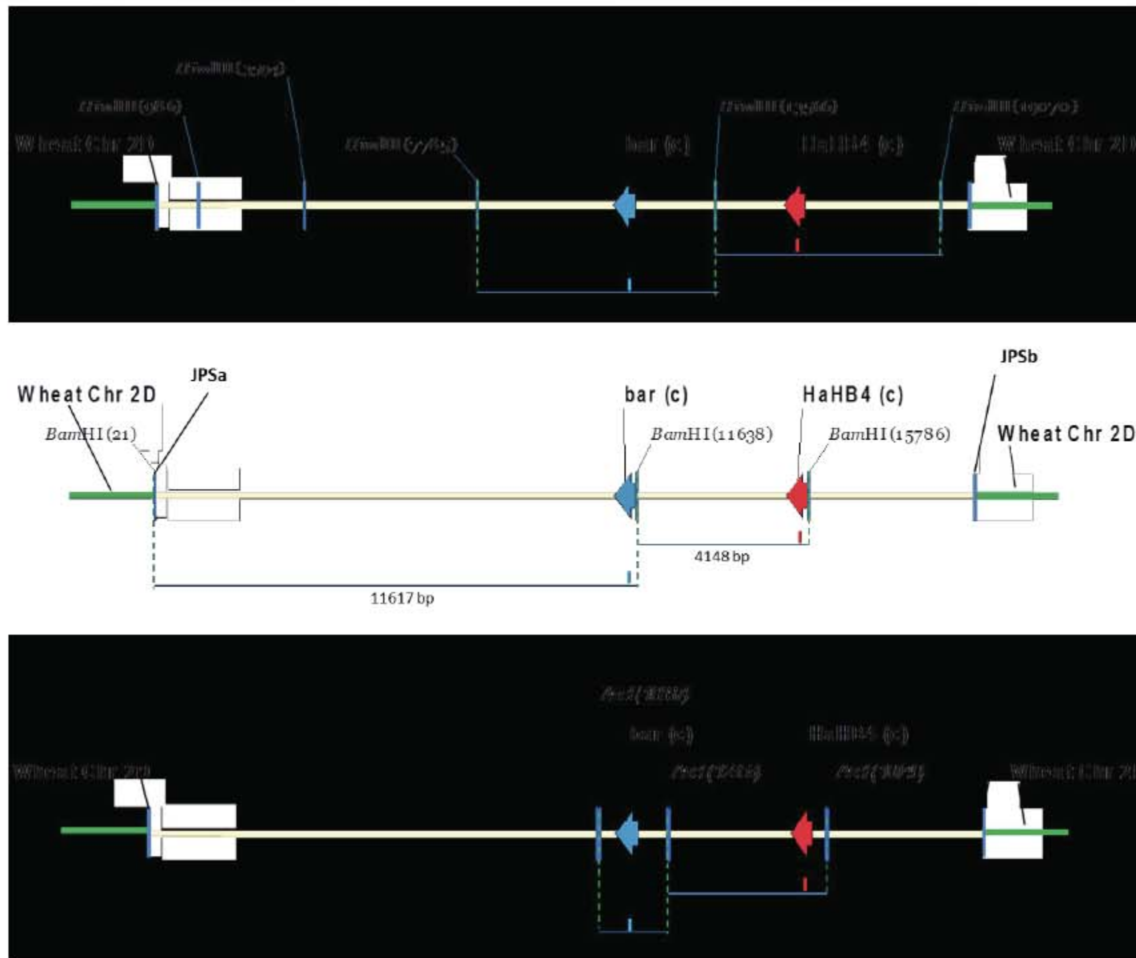

Figure 10. Schematic Representation of the *HaHB4* and *bar* Hybridising Fragments from the Short Insert Detected in the Southern Blot Hybridisation by Digestion with *Hind*III (a), *Bam*HI (b) and *Ase*I (c).

Solid arrows indicate CDSs of *HaHB4* (red) and *bar* (blue). The thick green line indicates the wheat genome. Below the insert, short vertical-coloured bars indicate the hybridisation sites for *HaHB4* (red) and *bar* (light blue) probes. Detected fragments are shown by thin horizontal blue lines with their size indicated underneath.

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2. HB4 wheat insertion sequence analysis

To overcome the complexity of resolving the insert structure, the Diversity Arrays Technology (DArT; Jaccoud et al., 2001; Wenzel et al., 2004) was used to identify the chromosome containing the insertion followed by comprehensive DNA sequence analysis using a combination of PacBio and Illumina reads (Zimin et al., 2017).

DArT allows the identification of an association between a desired locus (in this case, the *HaHB4* gene insertion) with molecular markers located within a chromosome. The basic assumption being that the gene and a chromosome-specific molecular marker would co-segregate when they are ligated in the same parental chromosome (Sun et al., 2016).

DArT technology was used to genotype the F2 generation of the crosses between HB4 wheat and a commercial wheat cultivar (IND-ØØ412-7 x Baguette 17), and to search for linkage of molecular markers with the *HaHB4* transgene. Each of the genomic samples (IND-ØØ412-7 and Baguette 17) were converted to 'representations' (Panel B in Figure 11), and the samples analysed (Panel C in Figure 11) were *HaHB4* positive and negative plants from the F2s.

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The chromosome containing the insertion was isolated from HB4 wheat by flow cytometry and assessed by two complementary sequencing strategies to determine the complete sequence of the insertion as well as the flanking sequences (Zimin et al., 2017; Figure 12). Firstly, Illumina sequencing was used to generate high-throughput short reads with a high coverage. To overcome the difficulties associated to the highly repetitive nature of the wheat genome, further sequencing was undertaken using PacBio sequencing that produces long reads and used as a complement to assist scaffolding of the Illumina reads.

A Junction Sequence Analysis (JSA) protocol (Kovalic et al., 2012) was applied using Illumina reads and identified up to 4 different flanking sequences with wheat DNA and several chimeric junction sequences involving plasmid elements. In brief, Illumina data also showed a complex pattern of the insertion event involving internal plasmid rearrangements in IND-ØØ412-7. Consequently, PacBio long sequencing reads were generated to assist the scaffolding process of the Illumina reads assembly. It was anticipated the PacBio long reads would allow reading through complex internal structures and rearrangements.

Combined analysis of sequencing data from Illumina and PacBio identified the flanking sequences of the insertion.

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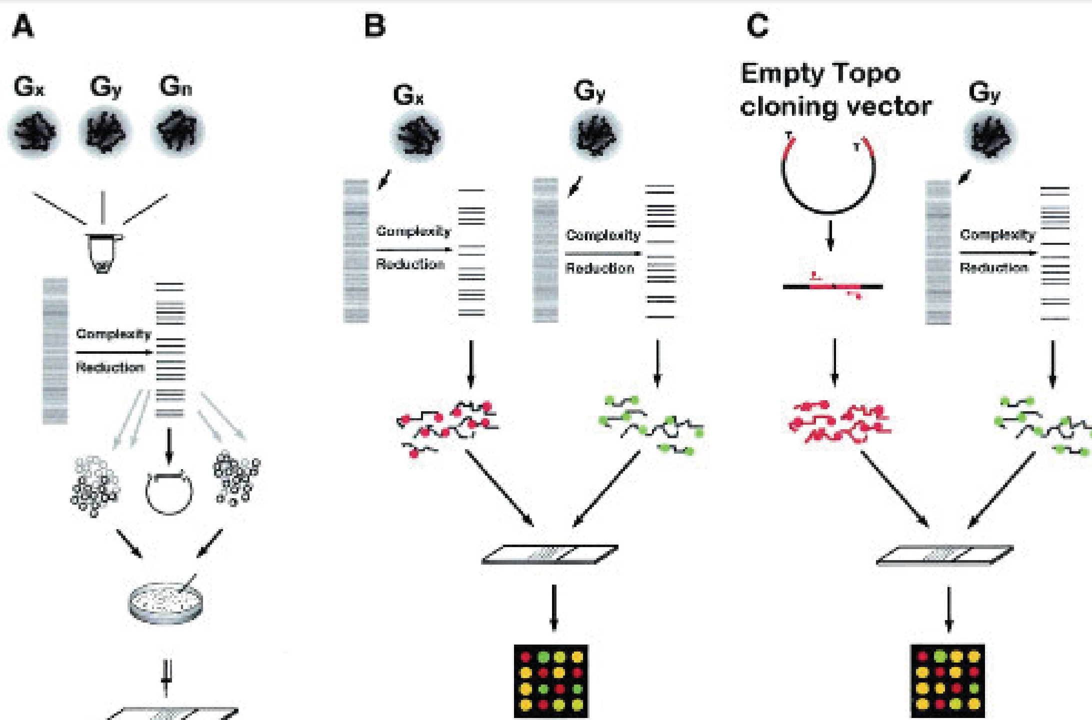

Figure 11. Schematic Representation of the Diversity Array Technology (DArT)
(**A**) Generation of Diversity Panels. Genomic DNA of specimens to be studied are pooled together. The DNA is cut with a chosen restriction enzyme and ligated to adapters. The genome complexity is reduced in this case by PCR using primers with selective overhangs. The fragments from representations are cloned. Cloned inserts are amplified using vector-specific primers, purified and arrayed onto a solid support. (**B**) Contrasting two samples using DArT. Two genomic samples are converted to representations using the same methods as in (A). Each representation is labelled with a green or red fluorescent dye, mixed and hybridised to the Diversity Panel. The ratio of green:red signal intensity is measured at each array feature. Significant differences in the signal ratio indicate array elements (and the relevant fragment of the genome) for which the two samples differ. (**C**) Genetic fingerprinting using DArT. The DNA sample for analysis is converted to a representation using the methods as in (A) and labelled with green fluorescent dye. Fragments of the cloning vector, which are common to all elements of the array (polylinker of PCR2.1-TOPO vector, marked red), are labelled with red fluorescent dye and hybridised to a Diversity Panel together with green fluorescence-labelled representation. First the ratio of signal intensity is measured at each array feature for each input genotype used to generate Diversity Panels. Polymorphic spots are identified by binary distribution of signal ratios among input samples. Any new specimen can be assayed on arrays of polymorphic features to generate a genetic fingerprint (Jaccoud et al., 2001).

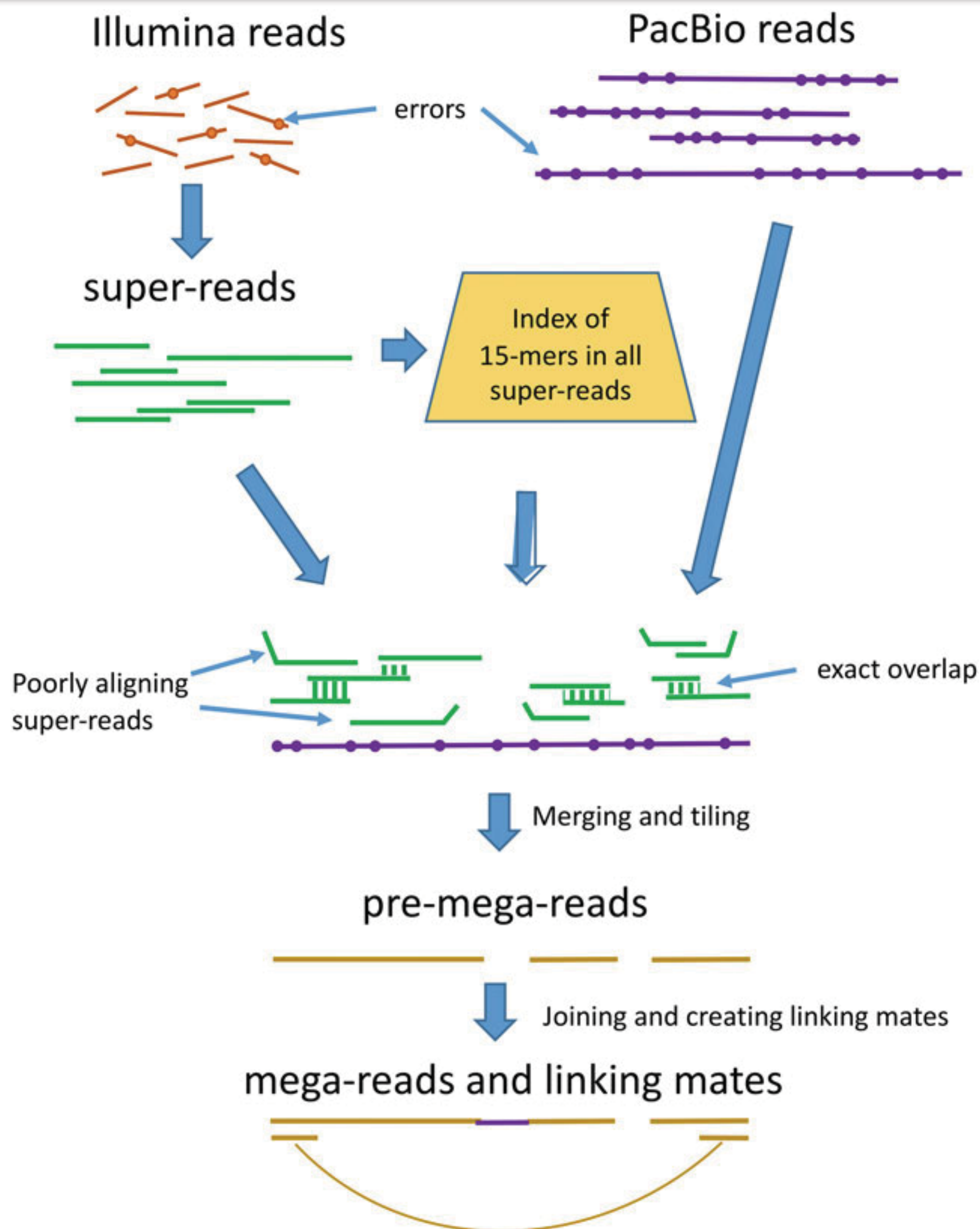

Figure 12. Overview of the mega-reads algorithm (from Zimin et al., 2017).
Low-error rate Illumina reads (top left) are used to build longer super-reads (green lines), which in turn are used to construct a database of all 15-mers in those reads. PacBio reads (purple lines) and super-reads are then aligned, using the 15-mer index. Inconsistent super-reads are shown as kinked lines; these are discarded, and the remaining super-reads are merged, using the PacBio read as a template, to produce pre-mega-reads (yellow). These are further merged to produce the final mega-reads and to generate linking mates across gaps

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A set of four parameters were established as a cut-off to determine the identity of a flanking sequence:

1. Supported by Illumina data
2. Supported by PacBio data
3. Supported by Illumina coverage as a single copy, and
4. Supported by PCR amplification from IND-ØØ412-7 DNA.

Four different flanking sequences passed all filters to meet the selected criteria suggesting two insertions. Each junction was named JPLa, JPLb, JPSa and JPSb, where JP stands for junction point for the large (JPL) or small (JPS) insert, and a and b refer to both sides of the inserts. A similar approach was used to determine the whole inserts sequences (see **Supplement Report A3c Molecular characterisation** for more details).

In brief, it was discovered that IND-ØØ412-7 event contains two different inserts integrated within a highly repetitive region of a chromosome in the wheat genome. One of the insertions is 47,611 bp long and the other is 20,418 bp long totaling 68,029 bp of insertions in IND-ØØ412-7 event (Figure 13 and Figure 14). The sequence of the insertions accounted for three copies of *HaHB4*, eight copies of *bar*, 19 copies of *bla* and four copies of the *gus* gene.

In the large insertion (Figure 13) it is possible to identify a complete *HaHB4* coding region (red) downstream of probable functional regulatory elements (Panel b). On the other hand, complete *bar*-coding regions (light blue) are observed in Panels a, b, c and d. In Panels (a) and (d) it is possible to identify that the regulatory elements of *bar* are complete and at the right position. These complete copies of *bar* potentially encode functional proteins. As a result, only one *HaHB4* copy and two *bar* copies with complete regulatory elements were identified.

In addition, backbone sequences of the plasmids are also observed, including the *bla* gene, a partial wheat *prGbl-1* promoter (Jones, 2005), *gus* CDS (Jefferson et al., 1987) and 35S-ter poly(A) signal from the Cauliflower Mosaic Virus 35S RNA (Benfey and Chua 1990). These sequences originated in a third plasmid used to monitor the efficiency of transformation, which have been incorporated during the integration process. The *gus* CDS was found in four copies while the *Gbl-1* promoter was found in six copies.

The complete sequence of the long and short inserts is provided in **Appendix 1** and the sequences of the Junction Points is Provided in **Appendix 2**.

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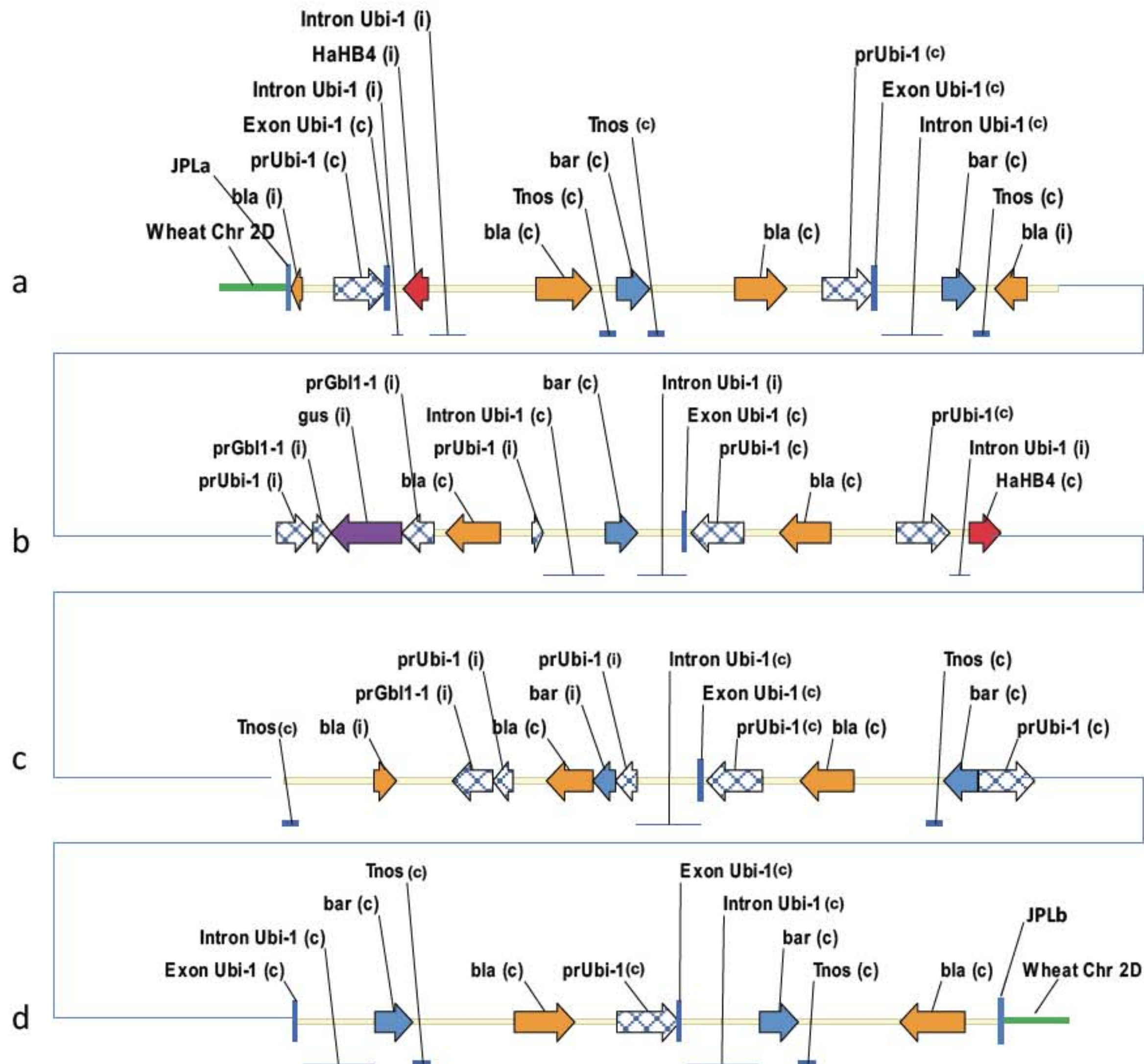

Figure 13. Scheme of the IND-ØØ412-7 Long Insert

This insert is subdivided in four fragments for clarity. **a)** *HaHB4* (red) incomplete (i) coding region is located between two incomplete fragments of *Ubi-1* intron. Besides there are two *bar* (light blue) coding regions, complete (c) and incomplete (i). The *bar* (i) is between two *Tnos* region. The *bar* (c) copy is located between its regulatory elements in the correct positions. **b)** *HaHB4* (c) coding region is located downstream of the proximal region of *Ubi-1* intron and the 5' promoter region and upstream its *Tnos. Bar* (i) is located between two *Intron Ubi-1* regions. **c)** *Bar* (c) is located between an invert *Ubi-1* promoter region and a *Tnos* region. *Bar* (i) is located between *Ubi-1* partial promoter region and *bla* gene. **d)** Two *bar* (c) coding region are located between their complete regulatory elements. Additionally, there were sequences of plasmids backbone, including *bla* gene (orange), partial *prGbl-1* promoter and *gus* CDS.

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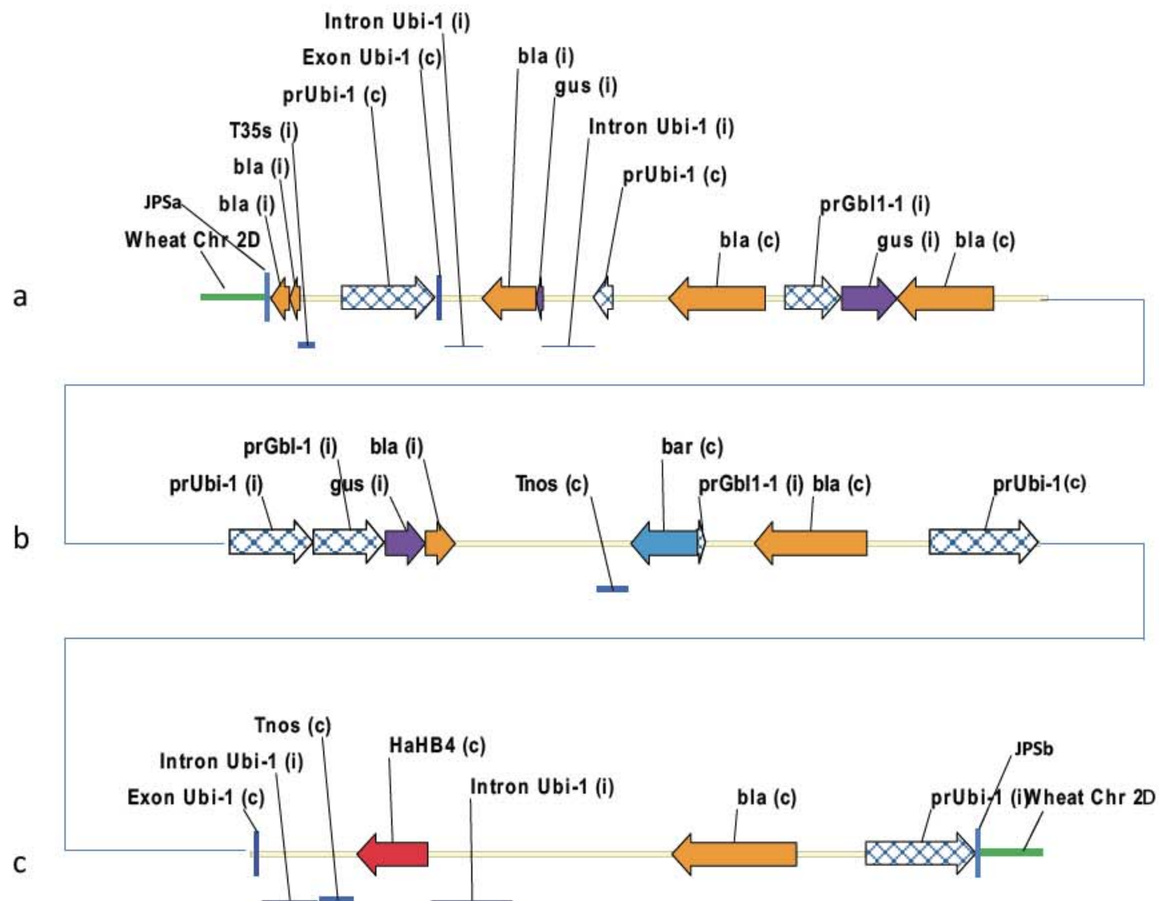

Figure 14. Scheme of the IND-ØØ412-7Short Insert

This insert is subdivided in three fragments for clarity. **a)** no *HaHB4* or *bar* elements are included in this part of the fragment. **b)** *Bar* (c) is located between an inverted and short sequence of *prGbl-1* promoter and a *Tnos* region. **c)** *HaHB4* complete (c) coding region is located between an incomplete *Ubi-1* intron and a *Tnos* element.

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3. Localisation of the HB4 wheat insert

A comparison of flanking sequences against the wheat genome revealed that the insertions were in a wheat chromosome region in which no annotated sequences were detected. Moreover, it is highly unlikely they were interrupting coding sequences.

All wheat sequences flanking the insert were DNA segments showed high similarity to retrotransposons, indicating insertion events within highly repetitive DNA regions (Table 6; Figure 15; Mayer *et al*, 2014). To confirm this, JPs were blasted against IWGSC and TGACv1 project wheat genomic databases through EnsemblPlants website (http://plants.ensembl.org/Triticum_aestivum/Info/Index). Multiple hits against several *T. aestivum* chromosome sites in both assemblies were observed in each case, although uneven in some cases (Figure 15). Another blast was performed against any other cereal genome available at EnsemblPlants database (*Triticum urartu*, *Aegilops tauschii* and *Hordeum vulgare*), resulting in the same multiple hit pattern against multiple chromosome sites. Sequences that are present in multiple chromosome sites and conserved in different organisms tend to be repetitive sequences.

JP sequences were also blasted against the NCBI non-redundant database. Two of these sequences (JP Short, JPSa and JPSb) matched against known retrotransposon elements (see Table 6). While the other two JP sequences (JP Long, JPLa and JPLb) had no significant hits against the NCBI database, they are highly conserved and repeated multiple times across multiple chromosomes in the wheat database, strongly suggesting unknown repeated DNA elements.

Table 6. Best Hit Example of JP Sequences BLAST[1] against NCBI Non-redundant Database

Query			Subject				Statistics			
Name	Start	End	Name	Start	End	Notes	Score	E-val	%ID	L
JPSa	1	266	FN564434	122244	122509	Retrotransposon gypsy	386	$1E^{-92}$	89	266
JPSb	1	499	AF326781	144816	145314	Transposon gypsy-like retrotransposon Fatima	582	$7E^{-146}$	83	499
JPLa	201	300	IWGSC_CSS_5AS scaff_1551050	364	463	unknow repeat element	500	$4E^{-15}$	100	100
JPLb	1	169	IWGSC_CSS_5DS scaff_2755707	2630	2797	unknow repeat element	507	$2E^{-15}$	81	174

1: Data accurate at the time of molecular characterisation
L.: Length

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Figure 15. Coverage Amongst JPs Against *T. aestivum*
High Scoring Pairs (HSP) plots are drawn for Blastn results against the TGACv1 reference assembly. **A**: JPLa; **B**: JPLb

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Figure 15 (continued). Coverage Amongst JPs Against *T. aestivum*
High Scoring Pairs (HSP) plots are drawn for Blastn results against the TGACv1 reference assembly. **C**: JPSa

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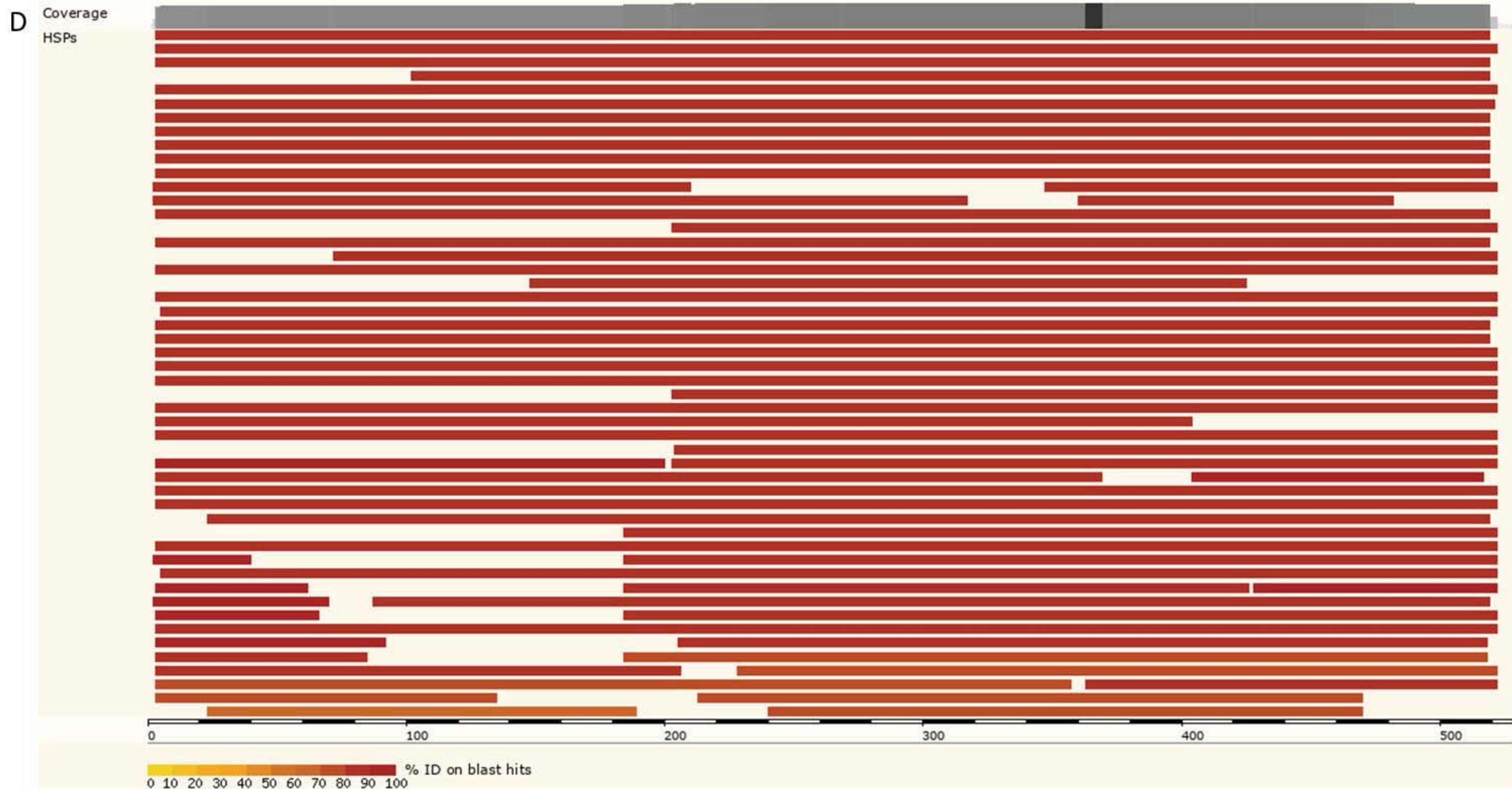

Figure 15 (continued). Coverage Amongst JPs Against *T. aestivum*
High Scoring Pairs (HSP) plots are drawn for BLASTn results against the TGACv1 reference assembly. **D**: JPSb

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4. Discussion and conclusions

Transgene integration into plant genomes, mediated by either *A. tumefaciens* or particle bombardment, is a random process that appears to correlate with the position of naturally occurring chromosome breaks (Altpeter et al., 2005). The delivery of whole plasmids by particle bombardment can lead to complex transformation events, in which the insertion of intact copies is accompanied by multiple, rearranged, and/or truncated fragments and vector backbone fragments, eventually interspersed by host genomic DNA sequences (Register et al., 1994; Pawlowski and Somers, 1996, 1998; Kohli et al., 2003).

Most transgene insertion events created by particle bombardment have the transgenic DNA integrated at a single locus (Breitler et al., 2002; Wilson et al., 2006). Although generally considered less prone to complex integration events, *A. tumefaciens*-mediated transformation also often results in multicopy T-DNA insertions (Heberle-Bors et al., 1988; Shou et al., 2004; Tzfira et al., 2004; De Buck et al., 2009; Oltmanns et al., 2010) or in profound insert or genome rearrangements (Nakano et al., 2005).

Integration of vector backbone accessory sequences in the plant genome is also a frequent phenomenon in *A. tumefaciens*-mediated transformation (Kononov et al., 1997; Wenck et al., 1997; De Buck et al., 2000; Lange et al., 2006). Interestingly, it has been found that breeding by conventional crosses is not necessarily more precise. In fact, introduction of unexpected changes and mutations in the plant genome through the breeding process may be deeper and wider compared with transgenesis (Batista et al., 2008). A noteworthy conclusion from the frequent occurrence of the above phenomena is that it has not resulted in undesirable consequences from the perspective of food or environmental safety of the products, as attested by the safe extended use of these transformation technologies. It is against this background that the genetic elements introduced into wheat event IND-ØØ412-7 was analysed.

Triticum aestivum has one of the most complex genomes known to science (Zimin et al., 2017; IWGSC 2018), with 6 copies of each chromosome, enormous numbers of near-identical sequences scattered throughout the genome (estimated at 80%; Mayer, 2014), and an overall haploid size of more than 15 Gbp (about 15 times the soybean genome, 40 times longer than rice and 5 times longer than the human genome). Both factors, the large size of its genome and the great extent of repetitive sequences pose great technical challenges in the genetic analysis of wheat event IND-ØØ412-7, making sequence alignment and assembly difficult, as they create ambiguities which, in turn, can produce biases and errors when interpreting results (Treangen and Salzberg, 2011). Classic methods that are normally used for the molecular characterisation of a transgenic event (e.g., insert copy number, insertion structure and genome location), such as Southern blot hybridisation, tail PCR, genome walking and sequencing, were found of limited value for the full characterisation of the integration locus in wheat event IND-ØØ412-7. Therefore, a specific strategy was designed to elucidate the insertion structure and sequence as outlined here and fully explained in the Supplement document.

Analysis of the insertion in wheat event IND-ØØ412-7 indicated a complex integration pattern consisting of two inserts located in one locus on chromosome 2D. Despite the complexity, the insertion generates functional HAHB4 and PAT proteins that provide tolerance to environmental stress and tolerance to glufosinate containing herbicides.

It must be emphasised that the complexity of the IND-ØØ412-7 insertion locus does not reflect or impact on food/feed and environmental safety, or on the expected inheritance pattern of this wheat event, as demonstrated through the extensive analysis presented in this dossier.

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A.3(c)(iv) A map depicting the organisation of the inserted genetic material at each insertion site

Details of the organisation of the inserted genetic material at each integration site are described above. Specifically:

HB4 Wheat–Detailed organisation of the genetic elements in each insert (Figure 13 and Figure 14).

A.3(c)(v) Details of an analysis of the insert and junction regions for the occurrence of any open reading frames (ORFs)

The sequence of both inserts and the wheat flanking regions were subjected to an ORF analysis (see Section B1(d) and the **Supplement Report B1d_Bioinformatic analysis**). None of the peptides that might be hypothetically produced from these ORFs were identified as homologs of known toxins or allergens (see Section B1(d)).

A.3(d) A description of how the line or strain from which food is derived was obtained from the original transformant (i.e. provide a family tree or describe the breeding process) including which generations have been used.

Particle bombardment is an established method for the introduction of plasmid vectors into plants. Wheat event IND-ØØ412-7 was developed using a particle bombardment procedure adapted from published methods (Barcelo and Lazzeri, 1995; Pastori et al., 2001; Rasco-Gaunt et al., 2001; Sparks and Jones, 2014). The process of development and selection event IND-ØØ412-7 is summarised in Figure 1 and detailed in the **Supplement Report A.3d Development of the HB4 wheat**.

A schematic representation of the development of HB4 wheat and the generations used for analysis is presented in Figure 16.

The original event IND-ØØ412-7 and its derivatives will continue to be crossed into elite wheat varieties through conventional breeding programs. Commercial varieties of wheat containing the HB4 trait will be used for food.

A.3(e) Evidence of the stability of the genetic changes, including:

(i) The pattern of inheritance of the transferred gene(s) and the number of generations over which this has been monitored
(ii) The pattern of inheritance and expression of the phenotype over several generations and, where appropriate, across different environments

A range of approaches were used to assess the stability of the insertion in HB4 wheat. Firstly, the event was crossed into a non-GM commercial wheat variety and the inheritance pattern of the GM trait evaluated. Further, the progeny of multiple generations obtained by self-pollination were tested for the presence of different elements associated to the IND-ØØ412-7 insertion. Also see the **Supplement Report A3c Molecular characterisation**.

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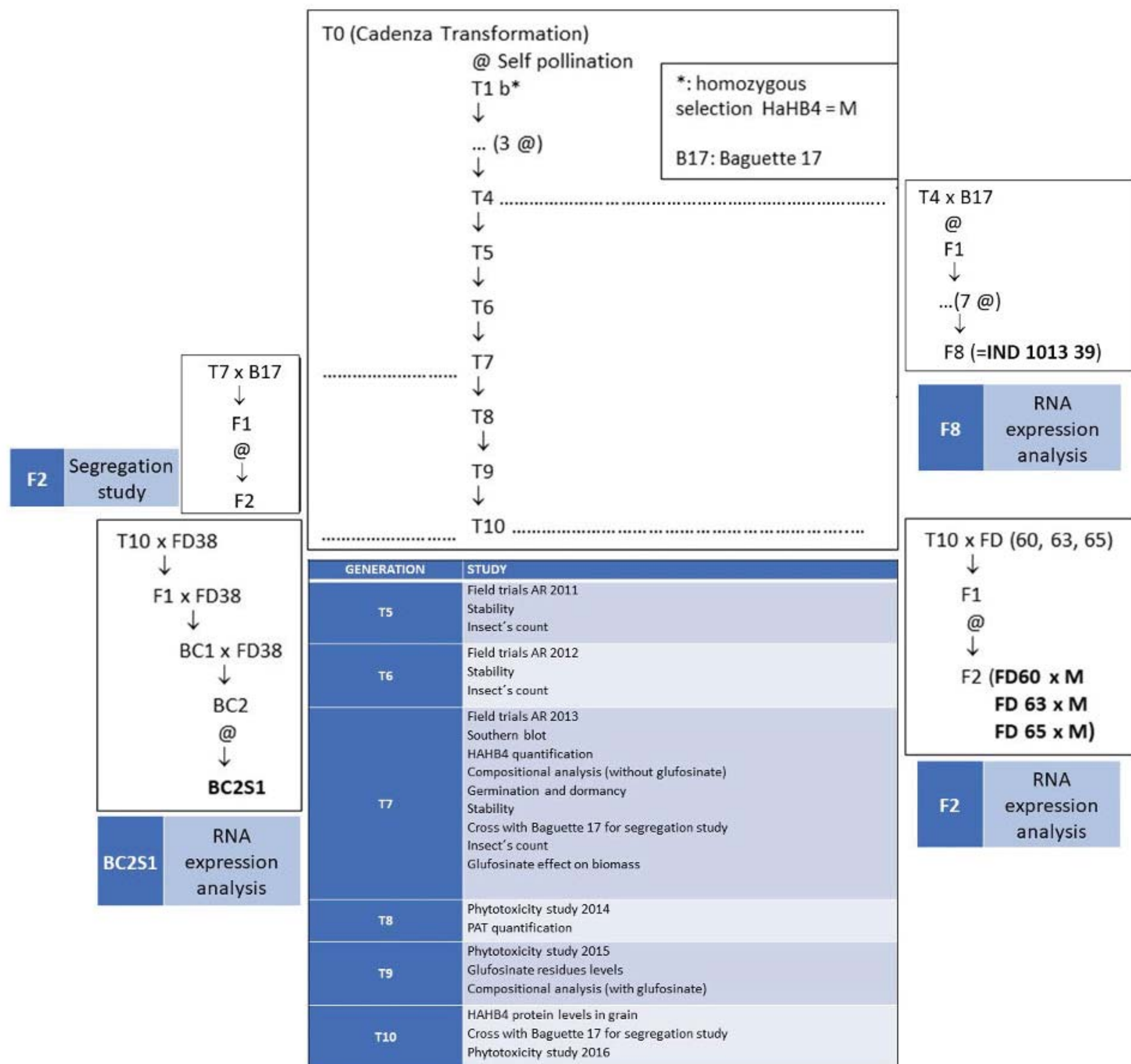

Figure 16. Schematic representation of the development of event IND-ØØ412-7 and the generations used in the different studies.

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Assessment of *HaHB4* and *bar* inheritance from HB4 Wheat

Homozygous T7 plants of event IND-ØØ412-7 were crossed with the non-GM commercial wheat variety Baguette 17. PCR analysis was undertaken for the presence of *HaHB4* and *bar* in F2 seedlings. The number of F2 plants that contained both *HaHB4* and *bar* were evaluated with respect to a 3:1 segregation ratio. Based on the analysis, the insertion in HB4 wheat is stably inherited and segregates in accordance with Mendel´s Laws.

An individual homozygous plant of HB4 wheat (T7 generation) was grown in a glasshouse alongside a non-GM plant commercial variety (Baguette 17). During flowering, the two plants were manually crossed, and all unused florets were emasculated to prevent self-pollination. This procedure generated forced cross seeds, termed F1 seed. The F1 seed were further grown to maturity and the flowers self-pollinated to generate a population of 349 F2 seeds. Each of the 349 seed were germinated and the seedlings analysed for the presence of the complete coding sequences *HaB4* and *bar* using end point PCR.

Chi-square goodness of fit tests were performed to assess segregation conformance to Mendelian inheritance.

Of the 349 F2 seeds produced from the IND-ØØ412-7 x Baguette 17 cross, 259 individuals showed the presence of both *HB4* and *bar*. A total of 90 plants were negative for both *HaB4* and *bar*.

Chi-square goodness of fit tests indicated that there was no significant departure from the predicted 3:1 segregation ratio (presence of *HB4* and *bar* X^2[1,N=349] = 0.11, P=0.74, p<0.05).

In addition to the analysis of the complete coding sequences of *HaHB4* and *bar*, the segregation study described above was extended to the characterisation of genetic elements within the insertion, including truncated *HaHB4* and *bar*; the *bla* gene, and the (four) flanking sequences. Therefore, the detection of *HaHB4*, *bar* and *bla* was analysed using oligonucleotides hybridising with both complete and incomplete copies of these elements. Other primers designed to detect the four insert-to-plant junctions (JPLa, JPLb, JPSa and JPSb, in Figure 13 and Figure 14, respectively) were also used to verify the stability of the insertion locus. All transgenic plants analysed in this extended approach presented results consistent with the co-segregation of all the genetic elements tested (*HaHB4*, *bar*, *bla* and for the four insert-to-plant junctions), That is they showed either the presence or the absence of all the seven genetic elements analysed.

A total of 92 individual F2 plants were assessed. Chi-square goodness of fit tests indicated that there was no significant departure from the predicted 3:1 segregation ratio (X^2[1,N=92] = 0.93, P=0.34, p<0.05).

Stability of the insertion in HB4 wheat

To verify the genetic stability of the insertion, an additional study based on the detection of the insert in plants form different generations was carried out. Genomic DNA isolated from leaf tissue was taken from seedlings of T5, T6 and T7 generations, and analysed by PCR using the same sets of oligonucleotides used for the segregation study. The presence of the several components of the inserts (*HaHB4*, *bar* and *bla* CDSs, complete or truncated), as well as the sequences of the insertion sites (JP), was analysed. All the samples analysed were positive for the complete set of elements, confirming the stability of the insertion locus.

Summary of genetic stability studies

The results of the segregation pattern in the F2 generation, as well as the presence of all the genetic elements in the different generations analysed support the conclusion that the IND-ØØ412-7 insertion resides at a single locus within the wheat genome, it is stable and is inherited according to Mendelian principles.

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A.3(g) An analysis of the expressed RNA transcripts, where RNA interference has been used

Not applicable to this application.

Conclusion of the Genetic Characterisation of event IND-ØØ412-7

A combination of DNA sequencing and Southern blot hybridisation were used to characterise the insertion in wheat event IND-ØØ412-7. The structure and sequences of the two inserts in HB4 wheat are provided, with flanking DNA sequence. Backbone DNA from the transformation vectors was also integrated into the Cadenza genome. No annotated genes were disrupted by the insertion in wheat event IND-ØØ412-7.

Studies have also confirmed the stability of the insertion in HB4 wheat across multiple generations.

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B. Characterisation and Safety Assessment of New Substances

B.1. Characterisation and Safety Assessment of New Substances

B.1(a) a full description of the biochemical function and phenotypic effects of all new substances (e.g. a protein or an untranslated RNA) that are expressed in the new GM organism, including their levels and site of accumulation, particularly in edible portions

The wheat event IND-ØØ412-7 was developed by transforming the wheat variety Cadenza, with the plasmid vectors *pIND4-HB4* and *pIND4-Bar* using particle bombardment. The event was developed to confer increased tolerance to environmental stresses avoiding reduction of crop yield and exhibits tolerance to glufosinate-based herbicides (Table 2).

Two new proteins are expressed in HB4 wheat; HaB4: the transcription factor HAHB4 that confers tolerance to environmental stress, and the enzyme phosphinothricin N-acetyl transferase (PAT), providing herbicide tolerance. Additional coding sequences for *gus* and the bacterial gene *bla* were also detected.

The *gus* and *bla* genes are derived from *Escherichia coli* and encode β-glucuronidase and β-lactamase respectively. The *gus* gene produces a blue stain on treated transformed tissue, which allows visual selection and the *bla* gene confers resistance to the antibiotic ampicillin.

The *gus* gene has a history of safe use (Gilissen et al. 1998) and several GM events contain the *gus* coding sequence have been approved by regulatory authorities, including FSANZ (e.g., Cotton Event MON-15985-7 (FSANZ A436); Soybean Event DD-026005-3 (FSANZ A387); and Sugar beet Event SY-GTSB77-7 (FSANZ A378)).

The *bla* gene is under the control of a bacterial promoter and was included as a marker to allow for selection of bacteria containing *pIND4-HB4* and *pIND4-Bar* prior to transformation of wheat. The *bla* gene has no plant regulatory sequences and is therefore unlikely to be expressed in plant tissues. Moreover, there are several GM events containing the *bla* coding sequence that have been approved by regulatory authorities, including FSANZ (e.g., Maize Event SYN-EV176-9 (FSANZ A385); Maize Event DKB-89614-9 (FSANZ A380); Maize Event ACS-ZMØØ3-2 (FSANZ A375) and Soybean Event DD-Ø26ØØ5-3 (FSANZ A387)).

Further information is provided in:

- **Supplement Report A1 Identity of genes and expression products**
- **Supplement Report B1a HAHB4 protein quantification in wheat seed**
- **Supplement Report B1a HAHB4 protein quantification in seedlings**
- **Supplement Report B1a PAT protein quantification.**

Identity and function of the HAHB4 protein

The homeodomain-leucine zipper (HD-Zip) gene family is an important class of transcription factors only found in plants (Henriksson et al., 2005; Ariel et al., 2007). Members of this gene family play vital roles in plant growth and development and participate in responding to various biotic and abiotic stresses (Liu et al., 2013; Li et al., 2019).

The *HaHB4* (*Helianthus annuus* homeobox 4) gene is a member of the HD-Zip sub-family I coding for the sunflower transcription factor HAHB4 (Dezar et al., 2005a; Harris et al., 2011; González et al., 2020).

Transgenic *Arabidopsis thaliana* plants expressing *HaHB4* exhibit a characteristic phenotype that includes a strong tolerance to water stress, are less sensitive to external ethylene and enter the senescence pathway later (Manavella et al., 2006). Expression studies in sunflower indicate that HaHB4 transcript levels are elevated in mature/senescent leaves and again demonstrated the action of this TF in the regulation of

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ethylene-related genes. Stable transformation of *Arabidopis* plants as well as transient transformation of sunflower leaves, further confirmed the involvement of HAHB4 in direct and indirect regulation of multiple stresses including water deficit, saline exposure, ABA and ethylene responses, photosynthesis, mechanical damage and herbivory. This and subsequent research (Manavella et al., 2008a, 2008b, 2008c, Dezar et al., 2005a, 2005b) led to the proposal that HAHB4 is involved in a novel conserved mechanism related to ethylene-mediated senescence and that this TF participates in the regulation of the expression of genes involved in developmental responses of plants to desiccation.

The sunflower HAHB4 protein was identified by using a degenerate oligonucleotide derived from the conserved HD amino acid sequence WFQNRRA to screen a cDNA library generated from sunflower stem (Chan and Gonzalez, 1994). HAHB4 was later shown to preferentially bind as a dimer to the dyad-symmetrical sequence CAAT(A/T)ATTG (Palena et al., 1999).

The amino acid sequence of the HAHB4 protein expressed in wheat event IND-ØØ412-7differs slightly from the one deduced from the nucleotide sequence of the cDNA of the mRNA transcript of the native sunflower HaHB4 gene that was annotated in the NCBI GenBank, Accession number AF339748.1 (Chan and Gonzalez, 1994; Gago et al., 2002; González et al., 2019; Figure 17). The differences include:

1. A deletion of amino acids 7-10 (as numbered by the NCBI original sequence, accession AAA63768.2).
2. A Lys to Arg substitution at position 22 (K22⇒R18)
3. A Phe to Leu substitution at position 159 (F159⇒L155)
4. A Phe to Leu substitution at position 175 (F175⇒L171)

```
      HAHB4   MSLQQVPTTETTTRKNRNEGRKRFTDKQISFLEYMFETQSRPELRMKHQL  50
  HAHB4Crop   MSLQQV----TTTRKNRNEGRRRFTDKQISFLEYMFETQSRPELRMKHQL  46

      HAHB4   AHKLGLHPRQVAIWFQNKRARSKSRQIEQEYNALKHNYETLASKSESLKK 100
  HAHB4Crop   AHKLGLHPRQVAIWFQNKRARSKSRQIEQEYNALKHNYETLASKSESLKK  96

      HAHB4   ENQALLNQLEVLRNVAEKHQEKTSSSGSGEESDDRFTNSPDVMFGQEMNV 150
  HAHB4Crop   ENQALLNQLEVLRNVAEKHQEKTSSSGSGEESDDRFTNSPDVMFGQEMNV 146

      HAHB4   PFCDGFAYFEEGNSLLEIEEQLPDPQKWWEF 181
  HAHB4Crop   PFCDGFAYLEEGNSLLEIEEQLPDLQKWWEF 177
```

Figure 17. Alignment of HAHB4 protein sequences
Alignment of the amino acid sequence of sunflower HAHB4 (Accession AAA63768.2) (HAHB4) and the sequence translated in IND-ØØ412-7 (HAHB4Crop). Numbers correspond to amino acid positions and are in frame with the GenBank HAHB4 accession

The introduction of *HaHB4* gene in wheat event IND-ØØ412-7 led to the environmental stress tolerance phenotype. Phenotypic and field performance evaluation of several HaHB4-containing lines allowed the selection of a transgenic wheat (termed IND-ØØ412-7), which was shown to provide an increased yield opportunity under conditions of environmental stress (González et al., 2019; González et al., 2020).

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HAHB4 is homologous to proteins with a history of safe use

Proteins with a history of safe use, or that are structurally and functionally related to proteins with a history of safe use, generally are considered safe to consume (Hammond and Cockburn, 2008). As a component of the safety assessment of HAHB4, bioinformatic analyses were conducted to identify sequence homology between the HAHB4 protein and proteins with a history of safe use.

HAHB4 is a member of the homeodomain-leucine zipper (HD-Zip) gene family and is found in sunflower and HB4 wheat. In 2015, the US Food and Drug Administration (FDA) has completed the Early Food Safety Evaluation (EFSE) process for HAHB4. In the EFSE process, the FDA reviewed safety data provided and supported the conclusion that the inadvertent presence of low levels of the HAHB4 protein would not raise food safety concerns (FDA 2015).

A thoughtful analysis of the HD-Zip superfamily performed by Harris et al. (2011), provides an unrooted phylogenetic tree of the HD-Zip protein superfamily. The tree contains over 50 selected sequences, grouped into clades (Figure 18).

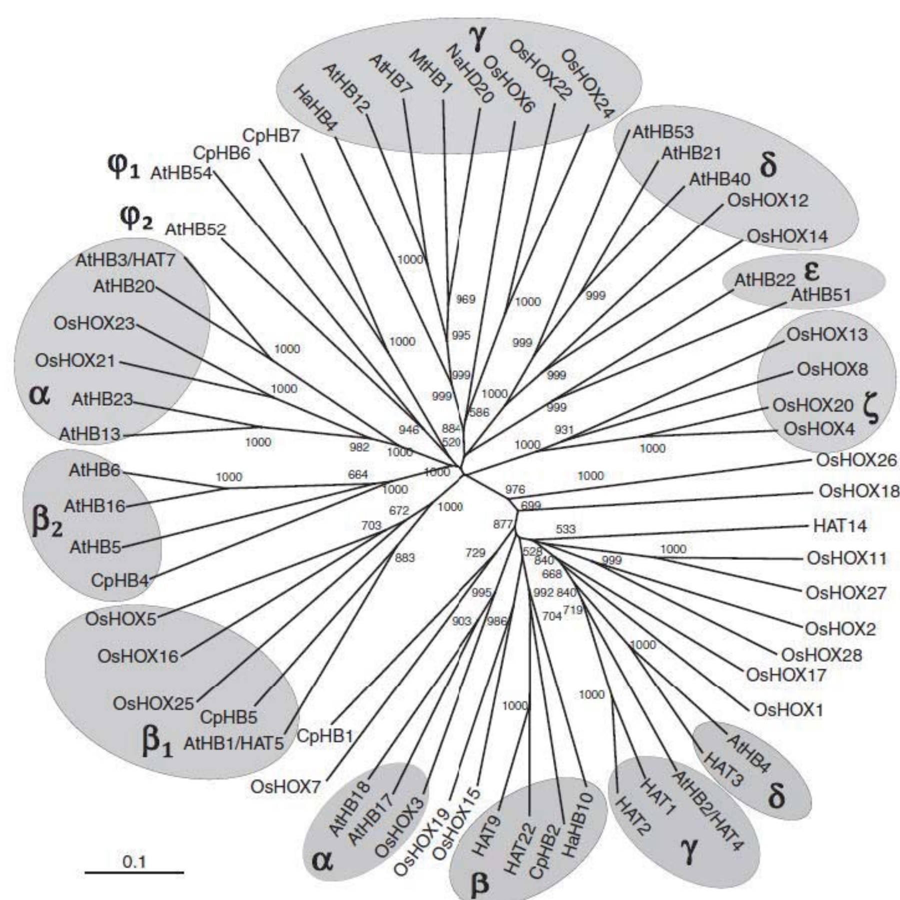

Figure 18. Unrooted phylogenetic tree of the HD-Zip protein superfamily

CLUSTALX alignment (Thompson et al., 1997) based on full-length amino acid sequences. The different clades within HD-Zip I and II family of proteins are circled and identified as α, β1, β2, γ, δ, ε, ζ, ϕ1 and ϕ2 (Agalou et al., 2008; Ciarbelli et al., 2008; Henriksson et al., 2005). Branch lengths are drawn to scale. Two-letter prefixes for sequence identifiers indicate species of origin. At, *Arabidopsis thaliana*; Cp, *Craterostigma plantagineum*; Mt, *Medicago truncatula*; Na, *Nicotiana attenuata*; Os, *Oryza sativa*; HB, homeobox; HOX, homeobox. Taken from Harris et al. (2011).

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Identity and function of the PAT protein

The *bar* gene in HB4 wheat is identical to that originally cloned from *Streptomyces hygroscopicus* (Murakami et al., 1986) and demonstrated to be useful as a selectable marker in other bacteria (Thompson et al., 1987) and in plants (Block et al., 1987). Importantly, the *bar* gene produces the enzyme phosphinothricin acetyl transferase (PAT), which breaks down phosphinothricin (also known as glufosinate), a broad-spectrum herbicide that acts as a competitive inhibitor of glutamine synthetase. As such, plants modified to contain the *bar* gene can tolerate herbicides that contain glufosinate ammonium.

Details on the common soil bacterium *Streptomyces hygroscopicus* are provided in Section A2(a)(i).

The PAT protein is expressed by several transgenic crops that have been in commercial production for many years. FSANZ have not identify any public health or safety concerns associated with the expression of PAT, as encoded by the *pat* or *bar* gene, in numerous assessments (for example, Soybean (A481, A1046, A1073, A1081); Canola (A372, A1140); Maize (A375, A380, A385, A386, A446, A543, A1106, A1116, A1118, A1192); Cotton, A518, A533, A1028, A1040, A1080); Rice (A589). The history of safe use of *S. hygroscopicus*, and safety data for the PAT protein are also provided in Herouet et al. (2005) and ILSI (2016). Therefore, this protein has been well characterised and demonstrated to be non-toxic to humans and animals.

HAHB4 Protein Expression in Wheat Event IND-ØØ412-7

Members of the HD-Zip family of transcription factors (TFs), unique to plants, have been shown to be involved in regulating the response of plants to environmental stress (Schena and Davis, 1992). TFs control gene expression by binding to genomic DNA in a sequence-specific manner.

Expression of genes of the HD-Zip subfamily I is regulated by external factors such as drought, extreme temperatures, osmotic stresses, and light conditions (Ariel et al., 2007; Chan, 2009). As such their expression levels under optimal growing conditions can be either non existing or extremely low (Suárez-López et al., 2001).

The *HaHB4* (*Helianthus annuus* homeobox 4) gene is a member of the HD-Zip sub-family I, coding for the sunflower transcription factor HAHB4 (González et al., 2020; see Section A.3(b)). The HAHB4 expression level in wheat event IND-ØØ412-7 proved to be too low to be measured using Western blot or ELISA methodologies. Therefore, a specific targeted LC-MS method based on HAHB4-specific proteotypic peptides was developed and validated using recombinant HAHB4 (**Supplement Report B1a HAHB4 quantification in wheat seed**).

Proteotypic peptides were detected and quantified using synthetic (heavy) peptide standards and isotope dilution. Stable isotope labelled peptides were used as internal standards and spiked into the sample to accurately quantify the endogenous levels of the transgenic protein. This workflow is like other targeted proteomic workflows for the identification of biomarkers and low-level endogenous proteins in complex matrices. The method was validated to be sensitive and accurate by fortification of control wheat flour with recombinant HAHB4 protein (rHAHB4; see **Supplement Report B2_Recombinant HB4 protein**). The detection methods demonstrated quantitative accuracy of between 86 and 103%. The limits of detection (LOD) and quantification (LOQ) for the HAHB4 protein assay were 0.01 and 0.03 µg/g DW seed, respectively. No HAHB4 protein was detected in any of the wheat seed samples analysed, obtained from field trials developed in three different locations of Argentina.

In separate experiments, the expression of HAHB4 protein in wheat event IND-ØØ412-7 was examined in plants exposed to osmotic stressors (NaCl, mannitol) in a growth chamber. Under these conditions and using equipment with greater sensitivity, HAHB4 protein in seedling extracts could be detected but the levels were below the lower limit of quantification (i.e., too low for accurate quantification). The highest value measured in this study was 0.018 ng/g FW (see **Supplement Report B1a HAHB4 quantification in wheat seedlings** for details). These observations confirm the very low level of HAHB4 protein in wheat event IND-ØØ412-7.

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PAT Expression in HB4 Wheat

The safety of PAT proteins has been well established. They are widely consumed since the very beginning of the development of genetically modified crops and shown not to raise concerns from a food/feed safety perspective (Hérouet *et al*, 2005; ILSI, 2016). As deduced from the construct and insert DNA sequencing, the amino acid sequence of the PAT protein in wheat event IND-ØØ412-7 is the same as previously assessed and deemed safe by FSANZ in other crops (e.g., Soybean: A481, A1046, A1073, A1081; Canola: A372, A1140; Maize: A375, A380, A385, A386, A446, A543, A1106, A1116, A1118, A1192; Cotton: A518, A533, A1028, A1040, A1080; and Rice A589).

The determination of the levels of PAT protein in wheat event IND-ØØ412-7 was determined using a commercially available ELISA kit, which was turned quantitative by the addition of a standard curve of recombinant PAT protein (See **Supplement Report B1a PAT protein quantification)**. Tissues samples taken from wheat IND-ØØ412-7 field trials at different developmental stages were used to quantify PAT protein expression (Table 7). Maximum levels found in seed were 3.79 µg/g of fresh weight, 11.55 µg/g in leaves and 12.67 µg/g in stems. The level of PAT protein in roots was below the LOD. These values are within the range (0.005 to 900 µg/g) reported in the literature for transgenic crops already approved for food and feed (ILSI, 2016).

B.1(b) Information about prior history of human consumption of the new substances, if any, or their similarity to substances previously consumed in food.

See the relevant parts of Section B.1(a) above on history of safe use and refer to the relevant supplemental reports. In addition, the dietary exposure of HAHB4 and PAT proteins to humans and livestock was estimated.

Low Dietary Exposure of HAHB4 and PAT protein to Humans in Australia and New Zealand

Data on the dietary consumption of wheat vary depending on the source and are difficult to estimate due to the numerous wheat-based products consumed. When considering wheat flour only, both Australia and New Zealand consumption rates are modest compared to other regions of the world (FAO 2018; Table 8).

The Australian Bureau of Statistics has estimated the median amount of flour, other cereal, and cereal products as well as cereal-based products and dishes consumed by Australians for various age groups. The largest daily consumption of wheat and wheat-based products are aged 14-18 (Table 9), with predominantly more consumption by males than females (Table 10). Data for New Zealand suggest that consumption of wheat flour is 25% greater than Australia (Table 8).

The potential dietary exposure of people to HAHB4 and PAT from consuming HB4 wheat was evaluated by calculating an estimate of daily dietary intake of HAHB4 and PAT for people and comparing this to daily protein intake (NHMRC 2006).

Dietary exposure calculations were determined using a conservative estimate of wheat flour consumption in New Zealand and Australian of 176kg and 135kg per capita per year, respectively (FAO 2018). An estimate of dietary exposure of humans to HAHB4 from wheat event IND-ØØ412-7 was calculated assuming wheat seed contain 0.01 µg/g (taken as the LOD) of HAHB4 and 4 µg/g of PAT, using wheat consumption data estimated in FAOSTAT (FAO 2018) and Australian Bureau of Statistics (43640DO006_20112012 Australian Health Survey: Nutrition First Results – Foods and Nutrients, 2011–12 — Australia).

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Table 7. PAT protein levels in HB4 wheat

Growth Stage	Tissue	Site*	Material (µg/g FW ± SE)**	
			HB4 Wheat	Cadenza
Tillering	Leaf	D13	10.11 ± 1.17	0
		A13	10.17 ± 0.48	0
		F13	***11.55 ± 0.94***	0
		H13	6.51 ± 0.72	0
		P13	11.06 ± 1.31	0
		I13	4.28 ± 0.30	0
Stem Elongation	Leaf	D13	7.11 ± 0.62	0
		A13	6.82 ± 0.24	0
		F13	6.61 ± 0.49	0
		H13	5.82 ± 0.23	0
		P13	11.36 ± 0.67	0
		I13	5.36 ± 0.59	0
Heading	Root	D13	0.00	0
		A13	0.00	0
		F13	0.00	0
		H13	0.00	0
		P13	0.00	0
		I13	0.00	0
Heading	Stem	D13	6.74 ± 0.65	0
		A13	8.72 ± 0.88	0
		F13	10.80 ± 0.71	0
		H13	6.59 ± 0.77	0
		P13	***12.67 ± 0.66***	0
		I13	5.36 ± 0.66	0
Maturity	Grain	D13	3.63 ± 0.50	0
		A13	***3.79 ± 0.35***	0
		F13	3.24 ± 0.32	0
		H13	2.11 ± 0.32	0
		P13	3.38 ± 0.40	0
		I13	1.78 ± 0.13	0

(*) Sites: Corral de Bustos (D13), Monte Buey (A13), Villa Saboya (F13), Daireaux (H13), San Jorge (P13) and Balcarce (I13)
(**) FW: Mean fresh weight from four independent replicates; SE: Standard Error.
Highlighted numbers represent the highest levels for the growth stage

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Table 8. Wheat Consumption for selected countries

Region	Wheat flour consumption (kg/capita/year)[1]
Azerbaijan	433
Egypt	324
Russian Federation	278
Argentina	240
United Kingdom	209
New Zealand	176
United States of America	162
Australia	135
Brazil	109
China	62

[1] Food and Agriculture Organization of the United Nations (FAO, 2018).

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Table 9. Median consumption of wheat-based foods in Australia[1]

Category (grams/day)	Age Group (persons in years)									
	2-3	4-8	9-13	14-18	19-30	31-50	51-70	71 and over	19 and over	Total 2 years and over
Cereals and cereal products	97.1	118.0	133.0	137.2	135.2	131.0	128.0	124.0	130.0	128.5
F ours and other cerea gra ns and starches	83.8	101.5	167.5	167.5	190.0	167.5	158.6	76.9	165.5	152.0
Regu ar breads, and bread ro s (p a n/unf ed/un topped var et es)	64.0	66.0	76.0	72.0	73.4	70.0	72.0	72.0	72.0	72.0
Eng sh sty e muff ns, f at breads, and savoury and sweet breads	53.6	67.0	71.6	72.0	71.0	71.0	67.0	67.0	71.0	71.0
Breakfast cerea s, ready to eat	25.5	34.0	39.0	53.0	56.1	50.0	45.0	34.0	47.8	43.0
Cereal-based products and dishes	98.5	124.8	203.4	247.6	255.1	192.2	130.0	71.6	175.0	175.0
Sweet b scu ts	19.5	22.4	30.0	32.5	34.9	25.2	24.0	25.2	26.4	26.0
Savoury b scu ts	14.0	19.0	25.0	41.7	19.0	19.8	19.0	14.2	19.0	19.8
Cakes, muff ns, scones, cake type desserts	*75.3	98.0	130.1	119.8	122.7	121.0	95.0	106.5	110.0	110.0
Pastr es	*76.9	130.0	130.0	171.9	145.6	140.0	137.5	133.9	140.0	135.0
M xed d shes where cerea s the major ngred ent	136.0	203.2	281.0	289.3	304.0	288.0	300.0	205.8	300.0	281.3
Batter based products	42.0	40.1	63.0	68.9	84.0	84.0	63.1	82.4	84.0	63.0

1: Data from 43640DO006_20112012 Australian Health Survey: Nutrition First Results – Foods and Nutrients, 2011–12 — Australia
* estimate has a relative standard error of 25% to 50% and should be used with caution.

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Table 10. Median consumption of bread wheat cereals and cereal based products[1]

Product category (grams per day)	Males		Females		Persons**
Cereals and cereal products	146.0		112.0		128.5
Flours and other cereal grains and starches	190.0		124.0		152.0
Gra ns (other than r ce) and gra n fract ons	38.4		39.3		38.6
Cerea f ours and starches	*125.6		*35.3		*63.0
Regular breads, and bread rolls (plain/unfilled/un-topped varieties)	82.0		66.0		72.0
Breads, and bread ro s, wh te, mandator y fort f ed	69.0		54.0		66.0
Breads, and bread ro s, wh te, add t ona vo untary fort f cat on	60.0		60.0		60.0
Breads, and bread ro s, wh te, not stated as to fort f cat on	64.0		64.0		64.0
Breads, and bread ro s, m xed gra n, mandator y fort f ed	60.0		60.0		60.0
Breads, and bread ro s, m xed gra n, add t ona vo untary fort f cat on	..		..		..
Breads, and bread ro s, m xed gra n, not stated as to fort f cat on	72.0		72.0		72.0
Breads, and bread ro s, who emea and brown, mandator y fort f ed	64.0		56.0		64.0
Breads, and bread ro s, who emea and brown, add t ona vo untary fort f cat on	46.0		*23.0		46.0
Breads, and bread ro s, who emea , not stated as to fort f cat on	66.0		66.0		66.0
English-style muffins, flat breads, and savoury and sweet breads	71.0		67.0		71.0
Eng sh-sty e muff ns	70.0		67.0		67.0
F at breads (e.g. P ta bread), wheat based	71.0		66.0		71.0
Savoury f ed or topped breads and bread ro s	85.0		85.0		85.0
Sweet breads, buns and scro s, un ced, unf ed	67.0		65.0		65.0
Sweet breads, buns and scro s, ced and/or f ed	85.0		80.0		85.0
Fr ed bread products and gar c breads	79.6		72.0		72.0
Breakfast cereals, ready to eat	51.0		35.0		43.0
Breakfast cerea , wheat based	51.0		34.0		34.0
Breakfast cerea , wheat based, fort f ed, sugars <=20 g/100g	51.0		34.0		34.0
Breakfast cerea , wheat based, fort f ed, sugars >20 g/100g	np		np		*27.9
Breakfast cerea , wheat based, w th fru t and/or nuts, unfort f ed	..		..		..
Breakfast cerea , wheat based, w th fru t and/or nuts, fort f ed, sugars <=25 g/100g	66.5		41.1		51.5
Breakfast cerea , wheat based, w th fru t and/or nuts, fort f ed, sugars >25 g/100g	*52.0		44.1		47.7
Breakfast cerea , m xed gra n	*54.6		*21.2		39.0
Breakfast cerea , m xed gra n, fort f ed, sugars <=20 g/100g	48.0		34.0		39.0
Breakfast cerea , m xed gra n, fort f ed, sugars >20 g/100g	39.0		36.2		39.0
Breakfast cerea , m xed gra n, w th fru t and/or nuts	84.6		58.8		70.5
Breakfast cerea , m xed gra n, w th fru t and/or nuts, fort f ed	54.6		43.2		48.8
Cereal based products and dishes	200.0		151.6		175.0
Sweet biscuits	32.0		22.4		26.0
Sweet b scu ts, p a n or f avoured nc ud ng short bread var et es	19.0		17.9		18.0
Sweet b scu ts, p a n w th fru t or nuts	20.0		21.0		20.2
Sweet b scu ts, w th jam, marshma ow or other sugar-based f ng	24.9		25.2		25.2
Sweet b scu ts, cream-f ed	32.0		25.0		29.4
Sweet b scu ts, choco ate-coated, choco ate ch p	34.0		29.6		34.0
Sweet b scu ts, choco ate-coated, choco ate or cream f ed	36.6		*18.3		36.6
Sweet b scu ts, other topp ngs	..		..		..
Savoury biscuits	22.1		18.9		19.8
Savoury b scu ts, wheat based, p a n, energy <=1800 kJ per 100 g	18.6		19.5		19.2
Savoury b scu ts, wheat based, p a n, energy >1800 kJ per 100 g	25.0		17.5		21.0
Cakes, muffins, scones, cake-type desserts	121.0		110.0		110.0
Cakes and cake m xes, choco ate	132.0		130.0		132.0
Cakes and cake m xes, sponge	125.0		*80.0		95.0
Cakes and cake m xes, other types	96.0		95.0		96.0
Muff ns, cake type, and muff n m xes	163.0		163.0		163.0

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Product category (grams per day)	Males	Females	Persons**
Cake-type desserts	75.0	90.0	75.0
S ces, b scu t and cake-type	80.0	55.9	65.2
Scones and rock cakes, p a n or w th added fru t or vegetab es on y	85.0	65.0	85.0
Scones and rock cakes, w th added cheese, choco ate or s m ar	np	*85.0	*85.0
Other desserts conta n ng cerea	np	np	*113.0
Pastries	155.2	130.0	135.0
Pastry, p a n/unf ed, a types	67.0	67.0	67.0
Sweet pastry products, fru t and/or nut f ngs	64.3	79.0	67.9
Sweet pastry products, egg or da ry based f ngs	100.0	76.7	95.0
Savoury pastry products, qu ches and f ans	135.0	135.0	135.0
Savoury pastry products, p es, ro s and enve opes	175.0	174.3	175.0
Savoury pastry products, p es, ro s and enve opes, fr ed	140.0	140.0	140.0
Mixed dishes where cereal is the major ingredient	305.8	230.0	281.3
P zza, saturated fat <=5 g/100 g	281.3	187.5	207.0
P zza, saturated fat >5 g/100 g	250.0	177.5	212.5
Sandw ches and f ed ro s, saturated fat <=5 g/100 g	263.1	157.5	196.0
Sandw ches and f ed ro s, saturated fat >5 g/100 g	*110.0	*183.0	183.0
Burgers, saturated fat <=5 g/100 g	256.6	210.0	236.3
Burgers, saturated fat >5 g/100 g	162.0	110.0	126.2
Batter-based products	72.7	59.5	63.0
Pancakes, crepes and d shes	68.0	66.2	68.0
Drop scones, p ke ets	*32.9	*23.2	*25.0
Waff es	96.8	np	*55.0
Batters and batter pudd ngs	75.2	np	*63.0
Doughnuts	63.0	63.0	63.0
Crumpets	84.0	62.0	84.0

1: Data from 43640DO006_20112012 Australian Health Survey: Nutrition First Results – Foods and Nutrients, 2011–12 — Australia
* estimate has a relative standard error of 25% to 50% and should be used with caution.
** Average of males and females

Based on FAO data, the Australian male population has a wheat flour consumption rate of 4.3 g/kg body weight/ day (g/kg bw/d) (Table 11). The highest consumers of wheat flour are New Zealand females at 6.5 g/kg bw/d. Based on this, the potential exposure of humans to HAHB4 and PAT protein from HB4 wheat was evaluated by calculating an estimate of daily dietary intake of HAHB4 and PAT consumption rates and comparing this to daily protein intake (Table 11 and Table 12). For these calculations, the average weights of Australian and New Zealand males and females was estimated based on data from the Australian Bureau of Statistics and Figures NZ data respectively.

Table 11. Dietary Exposure of Australian and New Zealand people to HAHB4 from HB4 wheat

Total Population	Gender	Wheat Consumption g/kg bw/d	Estimated Exposure to HAHB4 in Wheat Flour[1] μg/kg bw/d	Estimated Exposure to PAT in Wheat Flour[2] μg/kg bw/d
Australia*	Male	4.3	0.043	17.2
	Female	5.1	0.051	20.4
New Zealand**	Male	5.5	0.055	22.0
	Female	6.5	0.065	26.0

[1]Exposure to HAHB4 = (Consumption) × (0.01 μg HAHB4/g wheat grain), based on a conservative estimate at the LOD.
[2] Exposure to PAT = (Consumption) x (4.0 μg PAT/g wheat grain)
* Average weight of an Australian male, 87kg, and an Australian female, 72kg (ABS 2018)
** Average weight of a New Zealand male, 86.8kg and a New Zealand female, 74.5kg; Figure NZ (2020) Mean weight of adults in New Zealand. https://figure.nz/chart/Y1SYc1fTIqqbrvk8-BU1xLdNWfVykyuKu Accessed 28 April 2021

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Estimated exposure to HAHB4 protein in Australian males is 43 ng/kg bw/d and for females 51 ng/kg bw/d (Table 11). In addition, the percentage consumed compared to total protein intake is negligible (0.00001% for males and females; Table 12). Similar negligible exposure levels were estimated for the New Zealand population.

Estimated exposure to PAT protein in Australian males is 17.2 μg/kg bw/d and for females 20.4 μg/kg bw/d (Table 11). In addition, the percentage consumed compared to total protein intake is low (0.004% for males and 0.005% for females; Table 12). Similar low exposure levels were estimated for the New Zealand population.

Table 12. Dietary exposure of humans to HAHB4 and PAT as a proportion of daily protein intake

Total Population	Gender	Percentage of daily protein consumed that is HAHB4[1,2]	Percentage of daily protein consumed that is PAT[3,4]
Australia*	Male	0.00001%	0.004%
	Female	0.00001%	0.005%
New Zealand**	Male	0.00001%	0.006%
	Female	0.00002%	0.007%

[1]Exposure to HAHB4 = (Consumption) × (0.01 μg HAHB4/g wheat grain), based on a conservative estimate at LOD.
[2]Percent of daily protein consumed that is HAHB4 = (exposure to HAHB4/ 420 or 375 mg/kg bw/d).
[3]Exposure to PAT = (Consumption) x (4.0 μg PAT/g wheat grain)
[4]Percent of daily protein consumed that is PAT = (exposure to PAT/ 420 or 375 mg/kg bw/d).

The dietary exposure estimates are based on several assumptions. Firstly, it only considers wheat flour and assumes that 100% in the diet is derived from HB4 wheat. It also assumes that there is no degradation of HAHB4 or PAT proteins during milling or processing. Lastly, it also assumes that the proportion of protein consumed in the diet is 50% less than the recommended amount (NHMRC 2006), which results in an increased proportion of HAHB4 and PAT consumed in the diet.

These estimates indicate that dietary exposure to HAHB4 and PAT protein from HB4 wheat would be negligible.

Dietary Exposure of Livestock to HAHB4 and PAT

Wheat (*Triticum aestivum* L.) is generally thought as a grain crop, but it can be a useful winter pasture and forage source. The potential dietary exposure of cattle to HAHB4 and PAT from consuming HB4 wheat was evaluated by calculating an estimate of daily dietary intake of HAHB4 and PAT for cattle and comparing this to daily protein intake.

Grain is considered a cost-effective supplement for cattle. However, they need to be introduced to the diet gradually to avoid acidosis. A common wheat grain diet can consist of up to 6kg per day for Holstein-friesian dairy cows with a weight of between 550-600kg, however higher amounts have also been used.

The amount of protein in cattle feed varies depending on the diet and growth stage. An estimate of 15% protein in cattle feed on a dry matter basis (wheat grain) from HB4 wheat only, was used in the dietary exposure assessment and an average cattle weight of 580kg.

Using this information, the exposure estimates for HAHB4 and PAT consumed by cattle as grain was 0.1 μg/kg bw/d and 41.4 μg/kg bw/d respectively (Table 13 and Table 14). The estimate conservatively assumes that 100% of the wheat in a cow's diet is from HB4 wheat grain. When comparing the potential exposure of cattle to HAHB4 and PAT with the amount of total daily dietary protein, the percentage HAHB4 and PAT that cattle consume is negligible (approximately 0.0000065% and 0.003% respectively; Table 13 and Table 14).

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Table 13. Dietary Exposure Assessment of HAHB4 for cattle

Protein Source	Average Weight	Wheat Consumption[1]	Daily Protein Consumption (g/kg bw/d)[2]	Exposure to HAHB4 (µg/kg bw/d)[3]	Percent of Daily Protein Consumed that is HAHB4[4]
Wheat Grain	580 kg	6.0 kg/d	1.55	0.10	0.0000065%
Wheat Forage	580 kg	8.0 kg/d	2.1	0.14	0.0000067%

[1]Wheat consumption is based on a common diet of wheat grain or forage.
[2]Cattle daily protein consumption = {[(wheat consumption × 0.15) × 1000 g/kg] / 580 kg} assumes wheat is the only source of protein; for forage, based on 20Kg feed per day of which 40% is wheat dry matter
[3]Exposure to HAHB4 = (wheat consumption × 0.01 µg HAHB4/g wheat grain) / (average weight). Based on a conservative estimate of at LOD.
[4]Percent of daily protein consumed = (exposure to HAHB4 mg/ 1000 mg/g) / (daily protein consumption).

Table 14. Dietary Exposure Assessment of PAT for cattle

Protein Source	Average Weight	Wheat Consumption[1]	Daily Protein Consumption (g/kg bw/d)[2]	Exposure to PAT (mg/kg bw/d)[3]	Percent of Daily Protein Consumed that is PAT[4]
Wheat Grain	580 kg	6.0 kg/d	1.55	0.0414	0.003%
Wheat forage	580 kg	8.0 kg/d	2.1	0.138	0.007%

[1]Wheat consumption is based on a common diet of wheat grain or forage.
[2]Cattle daily protein consumption = {[(wheat consumption × 0.15) × 1000 g/kg] / 580 kg} assumes wheat is the only source of protein; for forage, based on 20Kg feed per day of which 40% is wheat dry matter
[3]Exposure to PAT in wheat grain = (wheat consumption × 4 µg PAT/g wheat grain) / (average weight); Exposure to PAT in wheat forage = (wheat consumption × 10 µg PAT/g wheat grain) / (average weight).
[4]Percent of daily protein consumed = (exposure to PAT mg/ 1000 mg/g) / (daily protein consumption).

Wheat can also be fed to animals as a fresh or dry forage. Cattle can consume up to 20kg of feed per day with a maximum of 40% of the forage intake the recommended inclusion rate of wheat forage in ruminant diets. Depending on when it is harvested, wheat forage can vary significantly in protein content. Wheat hay or straw can be quite low, up to 10% whilst fresh forage can be as high as 25% (Heuzé etal., 2015). For the purposes of the estimated exposure, protein content of 15% was used. Further, based on PAT protein estimates for vegetative tissue, a conservated value of 10 µg PAT/g in wheat forage was used (see Table 7).

Using this information, the exposure estimates for HAHB4 and PAT consumed by cattle as forage was 0.14 µg/kg bw/d and 138 µg/kg bw/d respectively (Table 13 and Table 14). The estimate also conservatively assumes that 100% of the wheat in a cow's forage diet is from HB4 wheat. When comparing the potential exposure of cattle to HAHB4 and PAT with the amount of total daily dietary protein, the percentage HAHB4 and PAT that cattle consume through forage is also negligible (approximately 0.0000067% and 0.007% respectively).

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In summary, the estimated exposure of humans and livestock consuming HB4 wheat containing the HAHB4 protein is negligible due to protein expression below the LOQ. Similarly, estimated exposure to PAT protein is also low.

Based on data from FAO, the Australian Bureau of Statistics and Figures NZ, calculations of Australia and New Zealand human consumption of HAHB4 and PAT protein from HB4 wheat would be negligible relative to daily protein intake.

Based on the maximum recommended incorporation rates, the consumption of HAHB4 and PAT protein from HB4 wheat would be a very small percentage of total protein consumed in livestock diets, even if HB4 wheat were to make up 100% of the wheat products consumed. Therefore, the exposure of humans and livestock to HAHB4 and PAT from HB4 wheat is negligible.

B.1(c) information on whether any new protein has undergone any unexpected post-translational modification in the new host

Glycosylation of proteins has been suggested as a distinguishing structural feature of allergenic proteins (Altmann 2007). Post-translational modifications (PTMs) to HAHB4 cannot be directly evaluated as protein expression levels are below the limit of detection. Further, the structure of the HAHB4 protein from wheat event IND-ØØ412-7 was searched for the signal sequence required for transport to the endoplasmic reticulum, a pre-requisite for glycosylation (Pattison and Amtmann et al., 2009) and other glycosylation sites. No such signal peptides were found in HAHB4 using the public algorithms SignalP-5.0 (Almagro Armenteros et al., 2019a), TargetP-2.0 (Almegro Armenteros et al., 2019b) and Predotar v1.3 (Small et al., 2004).

Additionally, glycosylation-acceptor sites were assessed using EnsembleGly software (Caragea et al., 2007; Gomord et al., 2010) and SPRINT-Gly (Taherzadeh et al., 2019). No consensus sequences for glycosylation were found.

The absence of both signal sequences for transport to ER and glycosylation acceptor sites suggests that glycosylation in HAHB4 from wheat event IND-ØØ412-7 is unlikely.

B.1(d) where any ORFs have been identified (in subparagraph A.3(c)(v) of this Guideline (3.5.1)), bioinformatics analyses to indicate the potential for allergenicity and toxicity of the ORFs

The sequence of the insertion in wheat event IND-ØØ412-7 was analysed in search of any open reading frame (ORF). Bioinformatic analysis were carried out to determine homology between known toxins or allergens and the hypothetical peptides that might be generated from these ORFs. A summary of the methods used to identify ORF sequences and evaluate the sequences against known allergens or toxins is provided in Table 15. Further details, including the sequence and location of the hypothetical peptides along the inserts and the procedures followed to carry out the different bioinformatic analysis are provided in the **Supplement Report B1d Bioinformatic analysis**.

In addition to the two new expression products expected, HAHB4 and PAT proteins, 67 putative peptides of 100 or more amino acids were found (summarised in Table 16). No relevant homology was found between these putative peptides to any allergenic or toxic sequence indicating that none of the hypothetical translation products derived from INDØØ412-7 wheat pose any safety concern.

Allergenicity Searches

Allergenicity potential was evaluated using the public, allergen-specific search engine (http://www.allergenonline.org/databasefasta.shtml) available through the Food Allergy Research and Resource Program (FARRP) at the University of Nebraska. All searches were performed using the most

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current database (version 21; February 14, 2021). Version 21 contains 2233 protein (amino acid) sequence entries that are categorised into 913 taxonomic-protein groups of unique proven or putative allergens (food, airway, venom/salivary and contact. The ORFs were analysed using full-length, 80-mer local, and 8-mer exact match alignments.

Table 15. Overview of analyses using bioinformatics

Analysis	Purpose	Approach
Start-to-stop ORF Analysis	Identify all open reading frames associated with the IND.ØØ412-7 wheat inserts, including flanking sequences.	Python script: systematically identify all ORFs (≥100 amino acids) located between a start codon and a stop codon where all six reading frames are considered (Biopython, 2018).
Allergenicity Analysis	Confirm that known allergenic sequences have not been generated by the genetic modification.	AllergenOnline (FASTA Search): identify any small regions of identity or larger regions of homology between ORFs and known allergens. Structural homology with allegens was also tested with SDAP
Toxicity Analysis	Confirm that sequences similar to known toxins have not been generated by the genetic modification.	BLAST (blastp) search: identify any ORFs with homology to proteins with "toxin" in its NCBI annotation. TADB2.0 (http://bioinfo-mml.sjtu.edu.cn/TADB2/) and T3DB (http://www.t3db.ca) were also searched.

In accordance with the globally recognised regulatory recommendations (FAO/WHO, 2001, Codex Alimentarius, 2003), the homology was analysed in fragments of 80 amino acids (Sliding 80mer window option) or identity of allergenic epitopes (8mer Exact Match).

To proceed with structural similarity analysis, the option "FASTA Search in SDAP" was selected on the Structural Database of Allergenic Proteins page (https://fermi.utmb.edu/SDAP/sdap_fas.html).

The analysis of potential allergenicity using the AllergenOnline database and tools confirmed no relevant homologies between the primary structure of the different amino acid sequences analysed with known allergens. This includes the absence of homology greater than or equal to 35% in 80 amino acids successive segments, as well as the absence of shared identity with allergenic epitopes when analysing successive peptides of 8 contiguous amino acids.

To detect a putative structural similarity with allergens, the SDAP database (Ivanciuc et al., 2002 and 2003) was used. No significant homology was found for any of the analysed sequences.

Toxicity Searches

To analyse potential toxicity of putative peptides, their homologous proteins, obtained from the alignment with the NCBI entries, was examined to search for the presence of any known toxin. Besides, similarity between putative peptides and the toxins grouped in TADB2 and T3DB databases was evaluated. No homology was found with known toxins. There was only one exception associated to PAT-associated peptides, which presented some new homologies with toxins (not existing in previous bioinformatic studies) (Figure 19). The homology found is related to the presence of a common N-acetyltransferase (NAT) domain, present in PAT and in a novel family of proteins belonging to the type II toxin-antitoxin systems having a GNAT (GCN5-related NAT)-fold (Jurenas et al., 2017). This toxin-antitoxin system was initially discovered in plasmids and its function is associated to plasmid maintenance in the growing bacteria population (Jurenas et al., 2017).

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Table 16. Summary of Putative Peptide Homologies with Proteins Registered in NCBI

		Short insert	Long insert	Total number of peptides
		Peptide number		
New expression product	HAHB4	6	12, 37	3
	PAT	8	17, 40-43, 46	7
Vector elements	Bla	2-4, 9, 17	3, 5-6, 10, 14, 22, 26, 28, 36, 44-45	16
	Gus	15	32	2
	Ubi-1	-	34	1
Without significant homology		5, 7, 11, 19	2, 7, 9, 11, 18-21, 23-25, 29-30, 35, 38-39, 47-48	22
Homology with cloning vectors		1, 10, 12, 14, 18	1, 4, 27, 31, 33	10
Hypotetical Protein from *Enterococcus faecium*		16	13, 15	3
Hypotetical protein from *Escherichia coli*		-	16	1
Hypothetical protein from wheat		13	-	1
Hypothetical protein from *Erwinia amylovora*		-	8	1
TOTAL				**67**

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Bioperl Reformatted HTML of BLASTP Search Report for PP_42

Gish, W. (1996-2006) http://blast.wustl.edu **Query=** PP_42 (Length: 229)

Database: TADB2_aa.fas

12,714 sequences; 1,560,970 total letters

Sequences producing significant alignments:	**Score (bits)**	**E value**
TADB|T5298 gi|194291114|ref|YP_002007021.1| phosphinothricin N-acety...	97.6	3.4e-24
TADB|T2094 gi|78065730|ref|YP_368499.1| N-acetyltransferase GCN5 [Bu...	76.2	1e-17
TADB|T4799 gi|161525329|ref|YP_001580341.1| N-acetyltransferase GCN5...	75.1	2.1e-17
TADB|T5352 gi|172060083|ref|YP_001807735.1| N-acetyltransferase GCN5...	74.4	3.4e-17
TADB|T2970 gi|115351078|ref|YP_772917.1| N-acetyltransferase GCN5 [B...	73.4	7e-17
TADB|T508 gi|17547852|ref|NP_521254.1| antibiotic resistance (acety...	70.9	3.9e-16
TADB|T838 gi|27376227|ref|NP_767756.1| phosphinothricin acetyltrans...	70.2	6.3e-16
TADB|T5113 gi|169633694|ref|YP_001707430.1| phosphinothricin N-acety...	67.7	3.5e-15
TADB|T5122 gi|169796191|ref|YP_001713984.1| phosphinothricin N-acety...	67.7	3.5e-15
TADB|T1353 gi|50084795|ref|YP_046305.1| phosphinothricin N-acetyltra...	66.7	7.3e-15
TADB|T2731 gi|107022223|ref|YP_620550.1| N-acetyltransferase GCN5 [B...	66.7	7.3e-15
TADB|T3104 gi|116689168|ref|YP_834791.1| GCN5-like N-acetyltransfera...	66.7	7.3e-15
TADB|T5233 gi|170732472|ref|YP_001764419.1| N-acetyltransferase GCN5...	64.5	3.1e-14
TADB|T1306 gi|53714970|ref|YP_100962.1| putative acetyltransferase [...	54.0	4.7e-11
TADB|T1765 gi|60682936|ref|YP_213080.1| putative acetyltransferase [...	54.0	4.7e-11

Figure 19. Toxicity Analysis with Toxin Antitoxin Database.
The complete amino acid sequence of each putative peptide was introduced into the search tool available in the "Toxin Antitoxin Database" (WU-BLAST 2.0). The result obtained for peptide 42 is shown as an example of the output obtained for the peptides associated to PAT protein. Upper panel: Alignment of peptide 42 with sequences in TADB. Lower panel: List of sequences with significant (E score < 10^{-5}) homology.

CONCLUSION

The performed analysis allowed the identification of putative expression products that could be generated by the genetic modification introduced in wheat event IND-ØØ412-7. The results of the ORF and the bioinformatic analysis included the new expressed proteins in HB4 wheat (i.e., HAHB4 and PAT).

The bio-informatic analysis demonstrated no relevant similarity between the putative peptides and known allergens or toxins.

Some homologies were found between PAT and a novel family of proteins belonging to the type II toxin-antitoxin systems since they possess a common NAT catalytic domain. This type of domain is known to be present in proteins from many species. For example, N-acetyltransferases catalyze the transfer of an acyl moiety from acyl coenzyme A (acyl-CoA) to a diverse group of substrates and are widely distributed in all domains of life (Salah Ud-Din et al., 2016). Furthermore, PAT protein safety has been established by scientific (Herouet et al., 2005) as well as regulatory precedents (CERA, 2011; ILSI, 2016). Besides, it is expressed in commercial GM crops approved in many countries (ISAAA, 2021), incorporated into glufosinate-tolerant crops since the very beginning of the GMO development (Stringam et al., 2003; CFIA, 1995). Based on the above, there is no evidence of a risk with the use of PAT protein in wheat event IND-ØØ412-7.

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Summary Safety assessment of HAHB4 protein

A weight-of-evidence approach using risk assessment principles was used to evaluate the safety of the HAHB4 protein.

The weight-of-evidence strongly supports HAHB4 safety:

- The prevalence of HD-Zip family of transcription factors in edible crops, including Sunflower (*Helianthus annuus*), is widespread in nature, and the HB4 protein is like proteins already present in the food supply with a history of safe consumption
- Bioinformatic analysis confirms that HAHB4 lack sequence similarity to known toxins and allergens (see below)
- Homology of HAHB4 to other proteins in plants with a history of safe use provides additional evidence that HAHB4 in wheat event IND-ØØ412-7 is as safe for human consumption as HD-Zip proteins like HAHB4 in other foods; and
- The potential exposure for humans and livestock to HAHB4 is negligible.

Based on the weight-of-evidence and considering the close-to-zero risk associated to the HAHB4 protein, wheat event IND-ØØ412-7 is as safe as conventional varieties for humans, livestock, and the environment.

Summary Safety Assessment of the PAT Protein

A weight-of-evidence approach using risk assessment principles was used to evaluate the safety of the PAT protein. This approach has been presented and assessed by FSANZ in numerous applications and considered all data in a comprehensive manner to evaluate the safety of PAT, including risk assessment results (potential hazard X potential exposure = potential risk).

As deduced from the construct and insert DNA sequencing, the amino acid sequence of the PAT protein in wheat event IND-ØØ412-7 is the same as previously assessed and deemed safe by FSANZ in other crops (e.g., Soybean: A481, A1046, A1073, A1081; Canola: A372, A1140; Maize: A375, A380, A385, A386, A446, A543, A1106, A1116, A1118, A1192; Cotton: A518, A533, A1028, A1040, A1080; and Rice A589).

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B.2. New Proteins

The PAT protein is identical to those previously assessed by FSANZ and an updated bioinformatics comparison of the amino acid sequence to known protein toxins, anti-nutrients and allergens is presented above in Section B1(d).

As HAHB4 has not been assessed by FSANZ previously, the following information is provided in accordance with the FSANZ Handbook.

B.2 (a) Information on potential toxicity

Details of the potential toxicity of the protein HAHB4 protein as well as other putative ORFs are presented in **Supplement Report B1d Bioinformatic analysis** and the following Sections:

- Section A.2(a)(i) and
- Section B.1(d)

The bio-informatic analysis demonstrated no relevant similarity between the putative peptides and known toxins. The HAHB4 protein is from sunflower and shares homology with numerous proteins found in food plants and therefore has a history of safe use.

B.2(a)(ii) information on the stability of the protein to proteolysis in appropriate gastrointestinal model systems

Details on the stability of the HAHB4 protein are provided in **Supplement Report B1b_Safety of HB4 protein.**

The HAHB4 protein is a transcription factor and present at extremely low concentrations in sunflower as well as in the wheat event IND-ØØ412-7. Therefore, isolation of sufficient quantities of protein from HB4 wheat was not feasible. Consequently, protein stability analysis was carried out using *E. coli* produced HAHB4 (rHAHB4), which proved to be equivalent to the native protein expressed in IND-ØØ412-7 wheat (see **Supplemental Report B2_Recombinant HB4 protein**).

Recombinant HAHB4 was subjected to simulated gastric fluid (SGF) assays performed following pre-established protocols (Thomas et al., 2004). Under these conditions, rHAHB4 protein was rapidly degraded as observed by the absence of the respective protein band 0.5 min after initiation of the assay (Figure 20). These results show that the HAHB4 protein is rapidly digested by pepsin *in vitro*.

B.2(a)(iii) an animal toxicity study if the bioinformatic comparison and biochemical studies indicate either a relationship with known protein toxins/anti-nutrients or resistance to proteolysis.

The bioinformatic analyses did not indicate any relationships with known protein toxins/anti-nutrients and did not show any resistance to proteolysis. However, a nutritional assessment was undertaken to compare HB4 wheat with the near isogenic parental line on broiler chicken performance (see **Supplement Report D Nutritional Study**). No significant differences were observed between wheat event IND-ØØ412-7 and its near-isogenic parental variety Cadenza, regarding zootechnic parameters and carcass characteristics.

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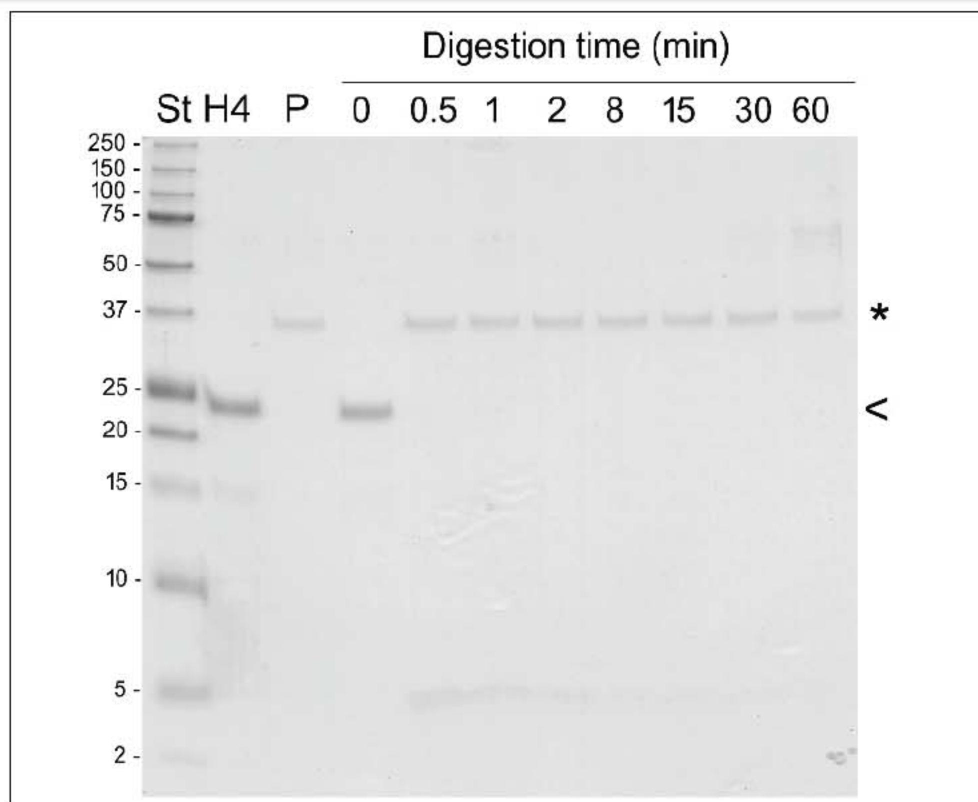

Figure 20. Digestibility of HAHB4

Recombinant HAHB4 (H4) incubated with pepsin (P) and analysed by SDS-PAGE and protein staining. <: indicates the location of the rHAHB4 protein band. *: indicates the location of the pepsin band. St indicates the molecular weight standard lane.

B.2(b) information on the potential allergenicity of any new proteins, including:

Details of the potential allergenicity of the protein HAHB4 protein as well as other putative ORFs are presented in **Supplement Report B1d Bioinformatic analysis** and the following Sections:

- Section A.2(a)(i) and
- Section B.1(d)

The bio-informatic analysis demonstrated no relevant similarity between the putative peptides and known allergens. The HAHB4 protein is from sunflower and shares homology with numerous proteins found in food plants and therefore has a history of safe use.

Additional information is provided below.

B.2(b)(iii) source of the new protein the new protein's structural properties, including, but not limited to, its susceptibility to enzymatic degradation (e.g. proteolysis), heat and/or acid stability

Details on the thermal stability of the HAHB4 protein are provided in **Supplement Report B1b_Safety of HB4 protein.**

A sample of rHAHB4 protein was incubated at different temperatures (60, 75 or 90 °C) for up to 60 min. Aliquots were taken after 10, 30 and 60 min of incubation and analysed by SDS-PAGE followed by protein staining (1.2 µg/lane) and ELISA. Results indicate that rHAHB4 integrity is not affected by heating. Incubation at 90 °C produced a slightly lower signal than the other tested temperatures even at short incubation times,

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but final absorbance values at 60 min did not show a significant difference from the control incubated at room temperature (Figure 21). These results suggest that the HAHB4 protein is not significantly degraded by high temperatures.

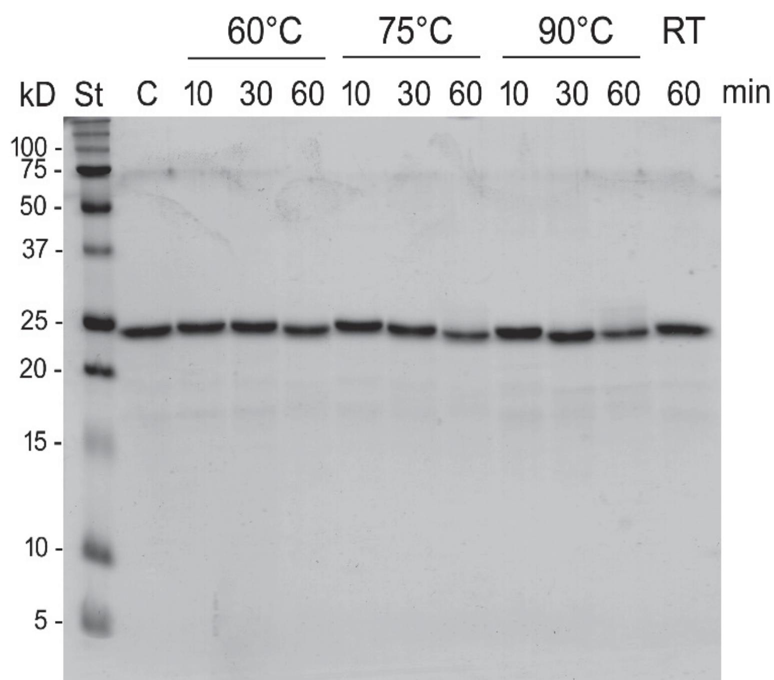

Figure 21. Effect of thermal treatment on rHAHB4 electrophoretic mobility.
rHAHB4 protein was incubated at different temperatures for up to 60 min and analysed by SDS-PAGE and protein staining. Original samples kept at 4 °C (C) or room temperature (RT) were included as controls. St indicates the molecular weight standard lane.

B.2(b)(iv) specific serum screening where a new protein is derived from a source known to be allergenic or has sequence homology with a known allergen

Not applicable. The HAHB4 protein is not from a source known to be allergenic nor does it display sequence homology with known allergens.

B.2(b)(v) information on whether the new protein(s) have a role in the elicitation of gluten-sensitive enteropathy, in cases where the introduced genetic material is obtained from wheat, rye, barley, oats, or related cereal grains.

Not applicable. The HAHB4 protein is not from wheat, rye, barley, oats or related cereal grains.

Where the new protein has been produced from an alternative source (e.g. microbial expression system) in order to obtain sufficient quantities for analysis, information **must** be provided to demonstrate that the protein tested is biochemically, structurally and functionally equivalent to that expressed in the food produced using gene technology.

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Details of rHAHB4 are provided in **Supplemental Report B2_Recombinant HB4 protein.** To ensure the recombinant protein was produced in *E. coli* as expected, HAHB4 was characterised for N-terminal sequence and protein mass analysis.

Protein purified from E. coli had the same sequence as the protein present in the IND-ØØ412-7 event (Figure 22). N-terminal sequencing of HAHB4 produced from the soluble and insoluble fractions confirmed no N terminal modifications, correct N-terminal amino acid sequence for the first seven amino acids (MSLQQVT) and confirmed the polyhistidine tag had been removed. Detection of the peptides from both the soluble and insoluble fractions demonstrated 47% coverage of the HAHB4 protein with each peptide scoring a probability greater than 90% that the sequence had been correctly identified. Taken together, the algorithms in Protein Prophet assigned a 99% probability the protein was correctly identified in the samples. Further, no HAHB4 peptides were present in the UniProtKB Wheat *Triticum aestivum* L. database ensuring the detected peptide sequences were only derived from HAHB4. MALDI-TOF analysis further showed the HAHB4 produced in *E. coli* were of the expected molecular mass. Based on the collective data from LC-MS analysis, MALDI-TOF mass detection, and N-terminal sequencing, HAHB4 protein produced in *E. coli* was shown to be equivalent to the protein present in IND-ØØ412-7 wheat. These data support the conclusion that HAHB4 protein is suitable for use in safety evaluations and to serve as a reliable standard for further studies.

```
ECOLI_HB4 MSLQQVTTTTRKNRNEGRRRFTDKQISFLEYMFETQSRPELRMKHQLAHKLGLHPRQVAIWFQNKRARSKSRQIEQEYNAL
412-7_HB4 MSLQQVTTTTRKNRNEGRRRFTDKQISFLEYMFETQSRPELRMKHQLAHKLGLHPRQVAIWFQNKRARSKSRQIEQEYNAL

ECOLI_HB4 KHNYETLASKSESLKKENQALLNQLEVLRNVAEKHQEKTSSSGSGEESDDRFTNSPDVMFGQEMNVPFCDGFAYLEEGNS
412-7_HB4 KHNYETLASKSESLKKENQALLNQLEVLRNVAEKHQEKTSSSGSGEESDDRFTNSPDVMFGQEMNVPFCDGFAYLEEGNS

ECOLI_HB4 LLEIEEQLPDLQKWWEF
412-7_HB4 LLEIEEQLPDLQKWWEF
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Figure 22. HAHB4 and rHAHB4 protein comparison
Sequence alignment showing the translated *E. coli*-produced HAHB4 sequence (ECOLI_HB4) is identical to the protein sequence translated from the plasmid transformed into IND-ØØ412-7 (412-7_HB4) and used to clone the gene sequence present in the E. coli expression vector pARC666.1 B8. As reported in the [Southern/sequencing data] section, the T-DNA sequence found in IND-ØØ412-7 is identical to the pIND4-HB4 plasmid.

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B.3. Other (non-protein) new substances

If other (non-protein) substances are produced as a result of the introduced DNA, information must be provided on the following:

B.3(a) the identity and biological function of the substance

B.3(b) whether the substance has previously been safely consumed in food

B.3(c) potential dietary exposure to the substance

Only two proteins are added the HB4 Wheat. The HAHB4 protein belongs to a large class of TFs unique to plants, which are associated to plant stress-response pathways. Therefore, being a component of the plant natural physiological response, no new proteins or metabolites other than the natural ones would be expected to arise from its activity.

B.3(d)(i) where RNA interference has been used: the role of any endogenous target gene and any changes to the food as a result of silencing that gene

Not applicable to this submission.

B.3(d)(ii) where RNA interference has been used: the expression levels of the RNA transcript

Not applicable to this submission.

B.3(d)(iii) where RNA interference has been used: the specificity of the RNA interference

Not applicable to this submission.

B.4. Novel herbicide metabolites in GM herbicide tolerant plants

The identity and levels of herbicide and any novel metabolites that may be present in the food produced using gene technology.

If novel metabolites are present then the application should address the following, where appropriate:

(a) toxicokinetics and metabolism

(b) acute toxicity

(c) short-term toxicity

(d) long-term toxicity and carcinogenicity

(e) reproductive and developmental toxicity

(f) genotoxicity.

The PAT enzyme is not anticipated to function within HB4 wheat any differently to the way that it functions within a range of other crops containing the same PAT enzyme and previously assessed by FSANZ (e.g., Soybean (A481, A1046, A1073, A1081); Canola (A372, A1140); Maize (A375, A380, A385, A386, A446, A543, A1106, A1116, A1118, A1192); Cotton, A518, A533, A1028, A1040, A1080); Rice (A589). Specifically, no novel metabolites would be expected to be formed and therefore, glufosinate-ammonium metabolism studies submitted to FSANZ previously in association with other crops are expected to sufficiently describe the metabolism of glufosinate-ammonium in HB4 wheat.

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B.5 Compositional analyses of the food produced using gene technology

This must include all of the following:

B.5(a) the levels of relevant key nutrients, toxicants and anti-nutrients in the food produced using gene technology compared with the levels in an appropriate comparator (usually the non-GM counterpart). A statistical analysis of the data must be provided.

B.5(b) information on the range of natural variation for each constituent measured to allow for assessment of biological significance should any statistically significant differences be identified

B.5(c) the levels of any other constituents that may potentially be influenced by the genetic modification, as a result, for example, of downstream metabolic effects, compared with the levels in an appropriate comparator as well as the range of natural variation.

> ***In the case of herbicide-tolerant plants, the levels of each constituent in the food produced using gene technology must be determined using plants sprayed with the herbicide.***

Verifying the compositional equivalence between genetically modified crops and their non-transgenic counterparts has been a main component in the safety evaluation of GM crops (Kuiper et al., 2001; Privalle et al., 2013). Following the outline of the OECD Consensus Document on Compositional Considerations for New Varieties of Wheat (OECD, 2003). Nutrients and micronutrients measured in grain (total 41 analytes) included proximates (moisture, protein, fat, ash, and carbohydrates), dietary fiber, minerals (calcium, iron, phosphorous, selenium and zinc), fatty acids (palmitic, stearic, oleic, linoleic, linoleic and arachidic) and amino acids (18) profiles and vitamins (thiamine, riboflavin, niacin, pyridoxine, folic acid and α-tocopherol). Two anti-nutrients were measured in grain: phytic acid and gliadin (43 total analytes in seed). Nutrients measured in forage (10 total analytes) included proximates, acid detergent fiber (ADF), neutral detergent fiber (NDF), dietary fiber and minerals (phosphorous and calcium). A similar separate compositional study was completed with the crop under glufosinate herbicide treatment, shown at the end of this sub-section.

Full details from compositional analysis of HB4 wheat are provided in **Supplement Report B5 Compositional analysis** and have been published Ayala et al., (2019). A summary of compositional analysis is presented below.

Samples (seeds and forage) were obtained from field trials conducted in Argentina during three growing seasons (2012, 2013 and 2015), at nine locations representing the environmental diversity over the range of the wheat producing regions. Field sites were distributed among three Provinces: Buenos Aires, Córdoba and Santa Fe. Samples were collected from the transgenic event IND-ØØ412-7, the parent non-transgenic variety Cadenza (SASA, 2014) as the near-isogenic comparator, and from five commercial reference varieties with desirable characteristics, currently in use in each region. The reference varieties were grown together to provide the range of natural variability of the crop, thereby giving the appropriate context for the interpretation of the experimental results in terms of the biological relevance. Grain samples were collected at maturity (2012 and 2015 trials), and forage samples were taken at tillering (2013 and 2015 trials).

Field trial results were analysed as a single group across all locations (combined site analysis) and for each site separately to determine whether there were significant compositional or nutritional differences between the transgenic event IND-ØØ412-7 and the parent non-transgenic variety Cadenza. The significant differences that might have been found were then analysed within the context of the range of values provided by the reference varieties and by the literature (See **Supplement Report B5 Compositional analysis**).

In a first set of analysis that involved samples from trials developed in 2012 and 2013, no significant differences were found in the levels of proximates, starch, dietary fiber, four minerals, and five vitamins when IND-ØØ412-7 wheat grain was compared to its non-transgenic parental line Cadenza (Table 17 and Table 18). Zinc and folic acid showed a significant difference with the control, but in both cases the levels

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measured in the transgenic wheat, although slightly below the control, were still within the range displayed by the local reference varieties and/or within those reported in literature. Statistically significant differences were also found in five of the six fatty acids analysed when levels measured in IND-ØØ412-7 wheat were compared to those found in the parental line (Table 17). However, in most of the cases, values were either within the range of values of the reference varieties and/or those reported in the literature. The only exception was the content of arachidic acid, which was greater in the event, and above the range provided by commercial varieties. However, in this case, values for both the wheat event IND-ØØ412-7 and Cadenza were below those of the reference varieties grown together, suggesting an effect due to the genetic background shared by both, the event and the parental control variety.

The analysis of the amino acid levels revealed that both the serine and threonine content were statistically higher in IND-ØØ412-7 wheat when compared to the parental control Cadenza (Table 18). However, threonine content was within the range of the local reference varieties, and the serine level was slightly above it, but within the literature range. No statistically significant differences between the transgenic event IND-ØØ412-7 and the parental control line Cadenza were found for the levels of the anti-nutrients gliadin and phytic acid (Table 19).

Overall, a compositional analysis of wheat grain materials taken from field trials involving glufosinate treatment (done at the 2015 season) also showed a similar picture of compositional equivalence to the parental control (Table 20 and Table 21). Only three significant differences were found between wheat IND-ØØ412-7 and its parental line. Protein and zinc levels in samples from glufosinate-treated plants were lower and leucine content in samples from plot without treatment were greater in the event when compared to Cadenza. However, in all these cases, values measured in the event were within reference ranges (Table 20 and Table 21).

Concerning forage nutrients, only three parameters (carbohydrate, moisture, and calcium) showed statistically significant differences when the levels measured in IND-ØØ412-7 wheat were compared to those of the parental control (Table 22). The carbohydrate and calcium contents fell within the range provided by the reference varieties. Moisture was slightly below this reference range.

In the analysis of forage from the glufosinate-treated wheat, only ash was found to be significantly different in IND-ØØ412-7 wheat irrespective of herbicide treatment (Table 23 and Table 24). However, the lower values measured in the event were within the reference range provided by the commercial varieties for both the herbicide-treated and untreated plants.

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Table 17. Summary of Differences for the Comparison of Wheat Components of IND-ØØ412-7 vs. Parental Control Line Cadenza

Components (Units)	Site	IND-ØØ412-7 Mean ± SE	Cadenza Mean ± SE	Difference (IND-ØØ412-7 minus Cadenza)	P	Commercial References Range	Literature Range
Statistical Differences Observed in Combined-Site Analysis							
Seed Fatty Acids (%tFA)							
Stearic Acid	-	1.68 (0.17)	1.39 (0.12)	0.29	<0.0001	1.86 - 2.05	0 - 4.6
Oleic Acid	-	20.36 (0.47)	19.41 (0.31)	0.95	0.0005	15.78 - 18.67	11 - 29
Linoleic Acid	-	56.87 (0.59)	58.51 (0.19)	-1.64	0.0001	56.59 – 59.05	44 - 74
Arachidic Acid		*0.91 (0.04)*	*0.86 (0.02)*	0.05	0.025	0.74 – 0.82	NA
Seed Minerals (ppm)							
Zinc	-	41.71 ± 1.89	45.75 ± 1.70	-4.04	0.001	32.03 – 34.87	24 - 47[1]
Seed Vitamins (ppm)							
Folic acid (B9)	-	0.29 ± 0.01	0.31 ± 0.01	-0.02	0.038	0.27 – 0.33	0.2 - 0.9[2]
Seed Aminoacids (% tP)							
Serine	-	3.45 ± 0.04	3.33 ± 0.03	0.12	0.011	3.25 – 3.43	4.3 – 5.7[2]
Threonine	-	2.58 ±0.02	2.48 ±0.02	0.10	0.004	2.43 – 2.58	2.4 – 3.2[2]
Forage Proximates							
Carbohydrates (% dw)	-	48.41 (1.37)	46.69 (1.38)	1.72	0.005	45.63 – 49.63	NA
Moisture (% fw)	-	*81.32 (0.24)*	*81.89 (0.27)*	-0.57	0.007	81.56 – 82.51	NA
Forage Minerals (% ps)							
Calcium	-	0.35 ± 0.01	*0.38 ± 0.01*	0.03	0.005	0.33 – 0.37	0.24[1]
Statistical Differences Observed in More Than One Site							
Seed Fatty Acids (% tFA)							
Stearic Acid	D12	1.85 ± 0.06	2.65 ± 013	-0.80	0.015	2.96 – 3.58	0 – 4.6[2]
	G12-1	1.42 ± 0.07	1.03 ± 0.04	0.39	0.011	1.76 – 2.20	
	H12	3.37 ± 0.13	1.18 ± 0.05	2.19	<0.001	1.65 – 2.01	

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Components (Units)	Site	IND-ØØ412-7 Mean ± SE	Cadenza Mean ± SE	Difference (IND-ØØ412-7 minus Cadenza)	P	Commercial References Range	Literature Range
Oleic Acid	D12	23.91 ±0.21	16.71 ±0.34	-1.21	0.001	20.70 – 27.19	11 – 29[2]
	I12	19.56 ± 0.20	20.77 ±0.10	7.20	0.011	15.18 – 16.90	
Linoleic Acid	D12	52.90 ± 0.55	59.47 ± 0.45	-6.57	0.007	48.55 – 53.61	44 – 74[2]
	H12	53.82 ± 1.22	57.88 ± 0.53	-4.06	0.036	57.51 – 60.37	
Linolenic Acid	G12-1	3.26 ± 0.08	3.90 ± 0.02	-0.64	0.003	3.42 – 4.32	0.7 – 4.4[2]
	H12	3.12 ± 0.09	3.30 ± 0.09	-0.18	0.044	3.13 – 3.81	
Arachidic Acid	A12-1	0.80 ± 0.04	*0.93 ± 0.04*	-0.13	0.016	0.77 – 0.88	NA
	D12	*1.22 ± 0.05*	*0.77 ± 0.05*	0.45	0.015	0.64 – 0.73	
	I12	0.72 ± 0.03	*0.85 ± 0.01*	-0.13	0.035	0.68 – 0.78	
Seed Minerals (ppm)							
Zinc	A12-1	47 ± 1	53 ± 1	-6	0.009	31 – 48	24 - 47[1]
	F12	45 ± 1	50 ± 1	-5	0.008	33 – 41	24 - 47[1]
	I12	24.25 ± 0.85	29.25 ± 0.48	-5	0.006	17.96 – 22.20	24 - 47[1]
Seed Vitamins (ppm)							
Riboflavin (B2)	*D12*	*0.28 ± 0.01*	0.54 ± 0.04	-0.26	0.015	0.29 – 0.41	0.6 – 3.1[2]
	G12-1	0.53 ± 0.02	*0.35 ± 0.02*	0.18	0.015	0.45 – 0.63	
Forage Proximates (% dw)							
Carbohydrates	H13	46.07 ± 0.51	43.44 ±0.86	2.63	0.006	42.06 – 51.20	NA
	P13	*57.42 ± 0.24*	51.17 ± 0.34	6.25	0.001	49.76 – 52.94	
Total Fat	A13	2.37± 0.22	2.75 ± 0.27	-0.38	0.038	1.78 – 3.01	NA
	F13	2.61 ± 0.18	2.07 ± 0.11	0.54	0.040	2.24 – 3.30	
Acid Detergent Fiber	H13	23.86 ± 0.38)	26.09 ± 0.52	-2.23	0.005	22.73 – 28.90	25.1 – 40.3[1]
	P13	25.15 ± 0.60	26.88 ± 0.45	-1.73	0.021	23.69 – 28.87	
Dietary Fiber	A13	14.74 ± 0.70	12.28 ± 0.19	2.46	0.026	11.25 – 15.06	NA
	P13	12.71 ± 0.36	*17.85 ± 0.58*	-5.14	0.008	11.14 – 14.00	

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Components (Units)	Site	IND-ØØ412-7 Mean ± SE	Cadenza Mean ± SE	Difference (IND-ØØ412-7 minus Cadenza)	P	Commercial References Range	Literature Range
Statistical Differences Observed in One Individual Site							
Seed Proximates (% dw)							
Total Protein	*A12-1*	*17.34 ± 0.24*	16.24 ± 0.14	1.10	0.006	15.59 – 16.83	10.0 – 16.0[2]
Seed Minerals (ppm)							
Phosphorus	H12	4873 ± 34	4960 ± 25	-87	0.009	4649 - 4985	3320 – 5160[1]
Seed Fatty Acids (% tFA)							
Palmitic Acid	G12-1	17.51 ± 0.34	16.97 ± 0.29	0.54	0.043	17.91 – 21.51	11 – 32[2]
Seed Vitamins (ppm)							
Tiamine (B1)	H12	4.13 ± 0.15	4.64 ± 0.15	-0.51	0.010	4.08 – 4.90	1.3 – 9.9[2]
Seed Amino Acids (% tP)							
Arginine	I12	4.29 ± 0.04	4.16 ± 0.01	0.13	0.018	3.90 – 4.39	4.0 - 5.7[2]
Leucine	F12	5.92 ± 0.17	6.48 ± 0.18	-0.56	0.013	6.71 – 6.92	5.0 – 7.3[2]
Serine	A12-1	*3.56 ± 0.17*	3.17 ± 0.10	0.39	0.033	3.13 – 3.48	4.3 – 5.7
Tryptophan	D12	1.66 ± 0.05	1.82 ± 0.05	-0.16	0.044	1.43 – 1.78	1.0 – 2.1[2]
Seed Anti-nutrients (% dw)							
Phytic Acid	*D12*	*1.73 ± 0.07*	1.26 ± 0.09	0.47	0.006	1.21 – 1.46	0.49 – 0.93[1]
Forage Proximates							
Moisture (% fw)	*H13*	*80.26 ± 0.33*	*82.30 ± 0.06*	-2.04	0.010	80.48 – 82.94	NA

SE: standard error of the mean; P: statistical significance; tP: total protein; tFA: total fatty acids; dw: dry weight; NA: not available; 1: ref. Orbert, 2004; 2: ref. OECD, 2003; Italics: indicate value out of range.

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Table 18. Summary of Wheat Seed Nutrients for IND-ØØ412-7 vs. Parental Control Line Cadenza. Combined Sites

Component (units)	IND-ØØ412-7 Mean (SE) (Range)	Cadenza Mean (SE) (Range)	Difference (IND-ØØ412-7 minus Cadenza)	Confidence Limits (95%)		Sig.	Commercial References Range	Literature Range[1]
Proximates (% dw)								
Ash	2.37 (0.09) (1.37 – 2.90)	2.32 (0.07) (1.69 – 2.79)	0.05	-0.17	0.27	NS	1.91 – 2.09	1.2 – 3.0
Carbohydrates	65.44 (0.50) (62.49 – 70.24)	65.76 (0.38) (63.02 – 70.28)	-0.32	-1.58	0.95	NS	65.42 – 67.52	65.4 – 78.0
Moisture (% fw)	13.09 (0.12) (12.14 – 14.75)	12.99 (0.16) (11.83 – 14.63)	0.10	-0.31	0.49	NS	13.99 – 14.30	8.0 – 18.0
Total Protein	*16.15 (0.40)* (12.33 – 18.43)	15.92 (0.32) (13.09 – 18.67)	0.23	-0.80	1.25	NS	14.23 – 15.16	10.0 – 16.0
Total Fat	2.26 (0.04) (1.82 – 2.61)	2.15 (0.06) (1.60 – 2.68)	0.11	-0.04	0.25	NS	2.13 – 2.29	1.5 – 2.0
Fiber (% dw)								
Dietary Fiber	13.79 (0.22) (11.95 – 15.48)	13.86 (0.24) (11.58 – 16.03)	-0.07	-0.72	0.59	NS	14.00 – 15.34	13.2 - 15
Minerals (% dw)								
Phosphorus	4912 (167) (3194 – 6146)	4961 (160) (3466 – 6061)	-49	-513	417	NS	3970 - 4534	3320 - 5160[2]
Calcium	461 (12) (373 – 573)	458 (12) (374 – 548)	3	-31	38	NS	441 – 501	250 – 538[2]
Iron	49 (2) (31 – 65)	50 (2) (30 – 76)	-1	-6	5	NS	38 - 43	33 - 79[2]
Selenium	0.55 (0.03) (0.35 – 0.78)	0.55 (0.03) (0.37 – 0.82)	0	-0.08	0.08	NS	0.53 – 0.58	0.04 – 0.71[2]
Zinc	42 (2) (22 – 63)	46 (2) (28 – 56)	-4	-9	1	*	32 - 35	24 - 47[2]
Fatty Acids (% tFA)								
16:0 Palmitic Acid	16.54 (0.21) (15.00 – 18.96)	16.20 (0.17) (14.96 – 17.67)	0.34	-0.21	0.87	NS	17.35 – 18.93	11 – 32
18:0 Stearic Acid	1.68 (0.17) (1.00 – 3.65)	1.39 (0.12) (0.95 – 2.88)	0.29	-0.13	0.71	*	1.86 – 2.05	0 – 4.6
18:1 Oleic Acid	20.36 (0.47) (17.78 – 24.33)	19.41 (0.31) (15.98 – 21.41)	0.95	-0.17	2.08	*	15.78 – 18.67	11 – 29

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Component (units)	IND-ØØ412-7 Mean (SE) (Range)	Cadenza Mean (SE) (Range)	Difference (IND-ØØ412-7 minus Cadenza)	Confidence Limits (95%)		Sig.	Commercial References Range	Literature Range[1]
18:2 Linoleic Acid	56.87 (0.59) (51.38 – 59.82)	58.51 (0.19) (56.37 – 60.66)	-1.64	-2.93	-0.37	*	56.59 – 59.05	44 – 74
18:3 Linolenic Acid	3.64 (0.09) (2.92 – 4.31)	3.62 (0.06) (3.05 – 4.19)	0.02	-0.19	0.24	NS	3.50 – 3.95	0.7 – 4.4
20:0 Arachidic Acid	*0.91 (0.04)* (0.65 – 1.32)	*0.86 (0.02)* (0.66 – 1.04)	0.05	-0.03	0.13	*	0.74 – 0.82	NA
Vitamins (mg/kg dw)								
Tiamine (B1)	4.02 (0.09) (3.10 – 4.67)	4.13 (0.10) (3.19 – 4.98)	-0.11	-0.38	0.16	NS	3.98 – 4.32	1.3 – 9.9
Riboflavin (B2)	*0.43 (0.03)* (0.25 – 0.81)	*0.40 (0.02)* (0.25 – 0.62)	0.03	-0.04	0.10	NS	0.48 – 0.66	0.6 – 3.1
Niacine (B3)	60.36 (2.19) (45.71 – 83.78)	58.78 (1.80) (46.73 – 80.88)	1.58	-4.13	7.29	NS	57.93 – 67.96	22.0 – 111.0
Pyridoxine (B6)	3.96 (0.09) (3.33 – 4.85)	4.07 (0.08) (3.31 – 4.79)	-0.11	-0.36	0.13	NS	3.94 – 4.23	0.9 – 7.9
Folic Acid (B9)	0.29 (0.01) (0.17 – 0.38)	0.31 (0.01) (0.16 – 0.40)	-0.02	-0.06	0.01	*	0.27 – 0.33	0.2 – 0.9
α-Tocopherol (E)	10.72 (0.39) (6.48 – 14.02)	10.61 (0.33) (7.66 – 13.73)	0.11	-0.92	1.14	NS	8.38 – 9.51	9 – 18
Amino Acids (% tP)								
Alanine	3.42 (0.04) (3.04 – 3.85)	3.48 (0.05) (3.19 – 4.33)	-0.06	-0.19	0.08	NS	3.22 – 3.51	3.4 – 3.7
Arginine	4.27 (0.04) (3.85 – 4.90)	4.21 (0.03) (4.03 – 4.58)	0.06	-0.03	0.15	NS	4.03 – 4.29	4.0 - 5.7
Aspartic Acid	5.08 (0.06) (4.32 – 5.66)	5.06 (0.03) (4.68 – 5.29)	0.02	-0.11	0.16	NS	5.01 – 5.13	4.8 – 5.6
Cysteine	2.60 (0.04) (2.40 – 3.09)	2.53 (0.02) (2.20 – 2.91)	0.07	-0.02	0.16	NS	2.50 - 2.59	1.7 – 2.7
Glycine	3.42 (0.03) (3.08 – 3.92)	3.41 (0.03) (3.17 – 3.94)	0.01	-0.09	0.10	NS	3.30 – 3.50	3.8 – 6.1
Glutamic Acid	27.43 (0.28) (24.92 – 29.58)	27.47 (0.36) (24.89 – 32.54)	-0.04	-0.96	0.87	NS	26.25 – 27.86	29.9 – 34.8
Histidine	2.57 (0.04)	2.54 (0.02)	0.03	-0.06	0.12	NS	2.48 – 2.58	2.0 – 2.8

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Component (units)	IND-ØØ412-7 Mean (SE) (Range)	Cadenza Mean (SE) (Range)	Difference (IND-ØØ412-7 minus Cadenza)	Confidence Limits (95%)		Sig.	Commercial References Range	Literature Range[1]
	(2.21 – 2.99)	(2.20 – 2.84)						
Isoleucine	3.39 (0.04) (2.79 – 3.81)	3.39 (0.03) (3.10 – 3.77)	0	-0.11	0.11	NS	3.27 – 3.45	3.0 - 4.3
Leucine	6.61 (0.09) (5.44 – 7.46)	6.72 (0.08) (5.71 – 7.71)	-0.11	-0.36	0.13	NS	6.66 – 6.85	5.0 – 7.3
Lysine	2.58 (0.03) (2.34 – 3.07)	2.56 (0.03) (2.20 – 2.93)	0.02	-0.07	0.12	NS	2.48 – 2.58	2.2 – 3.0
Methionine	1.65 (0.02) (1.40 – 1.78)	1.70 (0.03) (1.42 – 1.98)	-0.05	-0.11	0.02	NS	1.63 – 1.77	1.3 – 1.7
Phenylalanine	4.25 (0.03) (3.90 – 4.51)	4.21 (0.03) (3.74 – 4.56)	0.04	-0.05	0.12	NS	4.22 – 4.29	3.5 – 5.4
Proline	8.60 (0.06) (8.14 – 9.43)	8.50 (0.08) (7.34 – 9.29)	0.10	-0.10	0.30	NS	8.36 – 8.62	9.8 – 11.6
Serine	*3.45 (0.04)* (2.93 – 4.05)	3.33 (0.03) (2.94 – 3.55)	0.12	0.01	0.23	*	3.25 – 3.43	4.3 – 5.7
Threonine	2.58 (0.02) (2.49 – 2.90)	2.48 (0.02) (2.20 – 2.61)	0.10	0.04	0.15	*	2.43 – 2.58	2.4 – 3.2
Tryptophan	1.71 (0.02) (1.49 – 1.93)	1.69 (0.03) (1.39 – 2.05)	0.02	-0.04	0.09	NS	1.63 – 1.73	1.0 – 2.1
Tyrosine	2.55 (0.02) (2.29 – 2.80)	2.52 (0.03) (2.14 – 2.89)	0.03	-0.04	0.11	NS	2.40 – 2.58	1.8 – 3.7
Valine	4.25 (0.03) (3.88 – 4.51)	4.26 (0.04) (3.83 – 4.71)	-0.01	-0.11	0.08	NS	4.10 – 4.29	4.4 – 4.8

SE: standard error of the mean; Sig: statistical significance; NS: no significant difference; *: statistically different ($p<0.05$); dw: dry weight; fw: fresh weight; tFA: total fatty acids; tP: total protein; [1]: ref. OECD unless otherwise stated, 2003; [2]: ref. Obert, 2004; *Italics*: indicate value out of range.

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Table 19. Summary of Wheat Seed Anti-Nutrients for IND-ØØ412-7 vs. Parental Control Line Cadenza. Combined Sites

Component (% dw)	IND-ØØ412-7 Mean (SE) (Range)	Cadenza Mean (SE) (Range)	Difference (IND-ØØ412-7 minus Cadenza)	Confidence Limits (95%)		Sig.	Commercial References Range	Literature Range
Phytic acid	1.45 (0.05) (0.95 – 1.91)	1.47 (0.07) (0.97 – 2.04)	-0.02	-0.19	0.16	NS	1.31 – 1.46	0.49 – 0.93[1]
Gliadin	6.94 (0.21) (5.09 – 8.76)	7.06 (0.17) (5.67 – 8.52)	-0.12	-0.65	0.42	NS	5.93 – 6.75	3.9 – 9.1[2]

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Table 20. Summary of differences for the comparison of components of wheat IND-ØØ412-7 vs. Parental control line Cadenza with or without glufosinate

Component	Site	IND-ØØ412-7 Mean (SE)[e]	IND-ØØ412-7+G Mean (SE)	Cadenza Mean (SE)	P	Commercial Reference Range	Literature Range [f]
Statistical Differences observed in Combined-Site Analysis							
Seed Proximates (% dwt) [a]							
Protein		-	14.1 (0.2)	15.8 (0.4)	0.004	13.6 – 17.5	10.0 – 16.0
Seed Minerals (mg/kg dwt)							
Zinc		-	39 (3)	50 (3)	0.039	18 - 63	24 – 47
Seed Amino acids (% tP) [b]							
Leucine		7.11 (0.68)	-	6.07 (0.44)	0.043	4.70 – 8.20	5.0 – 7.3
Forage proximates (% dwt)							
Ash		14.62 (0.37)	14.50 (0.29)	15.61 (0.29)	0.009	10.68 – 16.68	NA
Statistical Differences observed in More than One Site							
Seed Proximates (% dwt)							
Carbohydrates [d]	PNO	68.2 (0.6)	69.1 (0.3)	66.4 (0.5)	0.010	66.4 – 69.5	65.4 – 78.0
	RLD	68.4 (0.7)	-	66.2 (0.2)	0.018	66.7 – 68.5	
Seed Vitamins (mg/kg dwt)							
Rivoflavin (B2)	MBY	0.35 (0.02)	0.36 (0.01)	0.68 (0.03)	<0.001	0.30 – 0.53	0.6 – 3.1
	PNO	0.65 (0.05)	-	0.25 (0.02)	0.002	0.31 – 0.50	
	RLD	0.69 (0.03)	0.72 (0.06)	0.37 (0.08)	0.004	0.24 – 0.57	
Seed Amino acids (% tP)							
Tryptophan	MBY	-	2.00 (0)	1.70 (0.11)	0.016	1.40 – 2.20	1.0 – 2.1
	PNO	1.83 (0.05)	1.93 (0.05)	2.65 (0.23)	0.004	1.40 – 2.70	

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Component	Site	IND-ØØ412-7 Mean (SE)[e]	IND-ØØ412-7+G Mean (SE)	Cadenza Mean (SE)	P	Commercial Reference Range	Literature Range [f]
Statistical Differences observed in One Individual Site							
Seed Proximates							
Moisture (% fwt) [c]	RLD	12.57 (0.08)	12.51 (0.11)	12.98 (0.10)	0.016	13.3 – 13.9	8.0 – 18.0
Seed Minerals (mg/kg dwt)							
Calcium	RLD	484 (7)	486 (10)	578 (15)	<0.001	454 - 574	250 - 538
Phosphorus	PNO	4630 (130)	-	3988 (69)	0.021	3368 - 3745	3320 - 5160
Seed Vitamins (mg/kg dwt)							
Niacin (B3)	MBY	54.7 (5.9)	52.8 (4.0)	77.3 (3.7)	0.008	59.5 – 66.4	22.0 – 111.0
Pyridoxine (B6)	MBY	3.7 (0.2)	4.4 (0.4)	6.9 (0.4)	<0.001	5.5 – 6.7	0.9 – 7.9
Seed Amino acids (% tP)							
Aspartic Acid	PNO	*6.15 (0.32)*	-	4.05 (0.17)	0.003	4.00 – 4.60	4.8 – 5.6
Histidine	RLD	2.13 (0.24)	-	3.20 (0.15)	0.003	2.80 – 3.30	2.0 – 2.8
Isoleucine	PNO	-	4.25 (0.23)	3.25 (0.24)	0.037	2.30 – 3.80	3.0 – 4.3
Lysine	PNO	*3.65 (0.36)*	-	2.20 (0.14)	0.002	2.00 – 2.70	2.2 – 3.0
Proline	RLD	9.83 (0.63)	-	7.38 (0.39)	0.016	6.90 – 9.50	9.8 – 11.6
Valine	PNO	4.18 (0.30)	-	3.23 (0.19)	0.039	2.40 – 3.50	4.4 – 4.8

[a] dwt = dry weight; [b] tP = total protein; [c] fwt = fresh weight; [d] determinate by calculation; [e] SE = standard error of the mean; [f] Ref. OECD, 2003; P = statistical significance ($p<0.05$); +G: with glufosinate; NA = not available; *Italics* indicates value out of range.

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Table 21. Summary of seed nutrients for wheat event IND-ØØ412-7 vs. Parental control line Cadenza Combined-sites. With and without glufosinate

Component	IND-ØØ412-7 Mean (SE)[f] Range	IND-ØØ412-7+G Mean (SE) Range	Cadenza Mean (SE) Range	Commercial Reference Range	Literature Range[g]
Proximates (% dwt) [a]					
Ash	2.50 (0.06) (2.29 - 3.03)	2.46 (0.06) (2.11 - 2.88)	2.46 (0.05) (2.17 - 2.71)	1.76 – 2.32	1.2 – 3.0
Carbohydrates [e]	68.0 (0.3) (66.4 - 69.5)	68.5 (0.3) (66.8 - 70.0)	66.8 (0.3) (65.4 - 68.2)	65.5 – 69.7	65.4 – 78.0
Moisture (% fwt) [b]	12.82 (0.10) (12.40 - 13.60)	12.73 (0.15) (12.07 - 13.60)	12.90 (0.13) (12.20 - 13.60)	15.7 – 13.9	8.0 – 18.0
Protein	14.9 (0.4) (12.7 - 16.5)	14.1 (0.2) * (12.3 - 15.1)	15.8 (0.4) (13.6 - 19.1)	13.6 – 17.5	10.0 – 16.0
Total Fat	1.5 (0.1) (1.1 - 1.7)	1.5 (0.1) (1.2 - 1.7)	1.5 (0.1 (1.3 - 2.2)	1.0 – 2.1	1.5 – 2.0
Fiber (% dwt)					
Dietary Fiber	13.1 (0.4) (11.7 - 16.5)	13.5 (0.3) (12.1 - 15.3)	13.4 (0.5) (11.1 - 16.1)	11.4 – 15.6	11.0 – 14.6
Minerals (mg/kg dwt)					
Calcium	453 (8) (403 - 501)	457 (12) (363 - 503)	483 (22) (396 - 621)	350 - 574	250 - 538
Iron	46 (2) (38 - 62)	45 (2) (38 - 62)	47 (2) (38 - 62)	31 - 44	33 - 79
Phosphorus	4620 (81) (4259 - 5247)	4573 (141) (3468 - 5104)	4438 (115) (3860 - 5068)	3368 - 5387	3320 - 5160
Selenium	0.30 (0.02) (0.15 - 0.45)	0.30 (0.02) (0.19 - 0.41)	0.30 (0.03) (0.15 - 0.49)	0.12 – 0.41	0.04 – 0.71
Zinc	42 (3) (26 - 63)	39 (3) * (24 - 55)	50 (3) (37 - 64)	18 - 63	24 - 47

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Component	IND-ØØ412-7 Mean (SE)[f] Range	IND-ØØ412-7+G Mean (SE) Range	Cadenza Mean (SE) Range	Commercial Reference Range	Literature Range[g]
Fatty Acids (% tFA) [c]					
Palmitic	15.9 (0.2) (14.9 - 17.5)	16.3 (0.2) (15.3 - 17.8)	15.6 (0.2) (13.8 - 16.7)	14.8 – 19.9	11 - 32
Stearic	1.5 (0.2) (1.0 - 2.8)	1.4 (0.2) (1.0 - 3.9)	1.3 (0.2) (1.0 - 2.8)	0.9 – 2.0	0 – 4.6
Oleic	20.4 (0.2) (19.5 - 22.8)	20.3 (0.3) (19.4 - 23.2)	20.9 (0.1) (20.3 - 22.0)	16.1 – 19.5	11 - 29
Linoleic	57.2 (0.5) (53.1 - 58.8)	57.3 (0.7) (51.3 - 59.3)	57.6 (0.4) (55.0 - 59.2)	55.5 -59.7	44 - 74
Linolenic	3.9 (0.1) (3.5 - 4.9)	3.7 (0.1) (3.2 - 4.3)	3.6 (0.1) (3.4 - 4.5)	3.6 – 5.4	0.7 – 4.4
Arachidic	1.0 (0.1) (0.7 - 1.3)	1.0 (0.1) (0.7 - 1.3)	1.0 (0.1) (0.7 - 1.3)	0.5 – 1.2	NA
Vitamins (mg/kg dwt)					
Thiamine (B1)	5.2 (0.3) (3.4 - 6.7)	5.3 (0.2) (4.1 - 6.3)	5.2 (0.3) (3.9 - 7.0)	4.0 – 7.4	1.3 – 9.9
Riboflavin (B2)	0.56 (0.05) (0.32 - 0.76)	0.47 (0.06) (0.20 - 0.84)	0.43 (0.06) (0.20 - 0.77)	0.24 – 0.57	0.6 – 3.1
Niacin (B3)	57.0 (3.1) (39.1 - 72.3)	63.8 (3.8) (45.1 - 90.4)	68.9 (2.8) (53.7 - 85.2)	59.5 – 73.2	22.0 – 111.0
Pyridoxine (B6)	4.2 (0.2) (3.3 - 5.7)	5.2 (0.3) (3.7 - 6.7)	6.2 (0.3) (3.8 - 7.7)	3.1 – 6.7	0.9 – 7.9
Folic Acid (B9)	0.16 (0.01) (0.13 - 0.19)	0.15 (0.01) (0.11 - 0.19)	0.15 (0.01) (0.11 - 0.19)	0.10 – 0.19	0.2 – 0.9
α-Tocopherol (E)	12.7 (0.8) (8.8 - 17.8)	14.8 (1.0) (9.2 - 18.2)	13.3 (0.9) (7.1 - 17.2)	7.0 – 14.4	9 - 18

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Component	IND-ØØ412-7 Mean (SE)[f] Range	IND-ØØ412-7+G Mean (SE) Range	Cadenza Mean (SE) Range	Commercial Reference Range	Literature Range[g]
Amino acids (% tP) [d]					
Alanine	3.31 (0.13) (2.30 - 4.20)	3.53 (0.13) (3.10 - 4.70)	3.60 (0.13) (2.70 - 4.40)	2.50 – 3.90	3.4 – 3.7
Arginine	3.87 (0.19) (2.70 - 5.30)	3.93 (0.15) (2.90 - 4.70)	3.88 (0.12) (2.90 - 4.40)	3.00 – 4.90	4.0 – 5.7
Aspartic Acid	5.36 (0.29) (4.20 - 7.00)	5.06 (0.18) (4.10 - 6.10)	4.75 (0.25) (3.70 - 6.50)	4.00 – 6.50	4.8 – 5.6
Cysteine	2.60 (0.13) (2.00 - 3.30)	2.77 (0.15) (2.20 - 3.70)	2.54 (0.16) (1.80 - 3.30)	2.10 – 3.60	1.7 – 2.7
Glycine	3.19 (0.23) (2.20 - 4.60)	3.33 (0.20) (1.80 - 4.40)	3.48 (0.18) (2.60 - 4.40)	2.70 – 4.00	3.8 – 6.1
Glutamic Acid	27.94 (0.34) (26.00 - 30.20)	28.87 (0.55) (25.80 - 32.10)	28.98 (0.68) (25.60 - 32.80)	24.70 – 32.70	29.9 – 34.8
Histidine	2.37 (0.10) (1.70 - 2.90)	2.88 (0.12) (2.20 - 3.40)	2.63 (0.16) (1.60 - 3.60)	2.00 – 3.30	2.0 – 2.8
Isoleucine	3.33 (0.12) (2.50 - 4.20)	3.48 (0.19) (2.60 - 4.80)	3.11 (0.16) (2.20 - 3.90)	2.30 – 3.80	3.0 – 4.3
Leucine	7.11 (0.68) * (3.80 - 9.50)	6.78 (0.59) (3.30 - 10.20)	6.07 (0.44) (3.90 - 8.50)	4.70 – 8.20	5.0 – 7.3
Lysine	3.14 (0.33) (1.40 - 4.70)	2.77 (0.35) (1.60 - 4.80)	2.85 (0.39) (1.70 - 5.40)	1.40 – 4.40	2.2 – 3.0
Methionine	1.83 (0.17) (0.90 - 2.90)	1.87 (0.19) (1.00 - 2.70)	1.59 (0.12) (1.10 - 2.30)	0.90 – 2.50	1.3 – 1.7
Phenylalanine	4.16 (0.26) (2.80 - 5.60)	4.31 (0.20) (3.00 - 5.40)	4.28 (0.18) (3.50 - 5.40)	2.70 – 6.20	3.5 – 5.4
Proline	8.86 (0.46) (6.60 - 11.70)	8.23 (0.26) (6.60 - 9.60)	7.73 (0.31) (6.50 - 9.60)	6.60 – 10.60	9.8 – 11.6

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Component	IND-ØØ412-7 Mean (SE)[f] Range	IND-ØØ412-7+G Mean (SE) Range	Cadenza Mean (SE) Range	Commercial Reference Range	Literature Range[g]
Serine	3.40 (0.13) (2.80 - 4.30)	3.40 (0.18) (2.30 - 4.60)	3.43 (0.22) (2.60 - 4.70)	2.10 – 5.80	4.3 – 5.7
Threonine	2.81 (0.24) (1.90 - 4.10)	2.43 (0.18) (1.50 - 3.50)	2.64 (0.22) (1.50 - 4.00)	1.60 – 3.60	2.4 - 3.2
Tryptophan	1.77 (0.02) (1.70 - 1.90)	1.85 (0.06) (1.40 - 2.00)	2.02 (0.16) (1.40 - 3.20)	1.40 – 2.70	1.0 – 2.1
Tyrosine	2.54 (0.17) (1.60 - 3.30)	2.61 (0.13) (1.90 - 3.30)	2.89 (0.11) (2.30 - 3.60)	1.90 – 2.90	1.8 – 3.7
Valine	3.43 (0.20) (2.70 - 5.00)	3.40 (0.10) (3.00 - 4.20)	3.17 (0.10) (2.60 - 3.60)	2.40 – 5.40	4.4 – 4.8

[a] dwt = dry weight; [b] fwt = fresh weight; [c] tFA = total fatty acid; [d] tP = total protein; [e] determinate by calculation; [f] SE = standard error of the mean; [g] Ref. OECD, 2003; +G: with glufosinate; *statistical difference ($p<0.05$); NA = not available; *Italics* indicates value out of range.

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Table 22. Summary of Wheat Forage Nutrients for IND-ØØ412-7 vs. Parental Control Line Cadenza. Combined Sites

Component (units)	IND-ØØ412-7 Mean (SE) (Range)	Cadenza Mean (SE) (Range)	Difference (IND-ØØ412-7 minus Cadenza)	Confidence Limits (95%)		Sig.	Commercial References Range	Literature Range[1]
Proximates (% dw)								
Ash	*11.24 (0.26)* (11.04 – 11.45)	11.64 (0.20) (11.34 – 11.95)	-0.40	-1.05	0.25	NS	11.27 – 12.79	NA
Carbohydrates	48.41 (1.37) (47.58 – 49.17)	46.69 (1.38) (45.81 – 47.78)	1.72	-2.19	5.63	*	45.63 – 49.63	NA
Moisture (% fw)	*81.32 (0.24)* (81.13 – 81.71)	*81.89 (0.27)* (81.54 – 82.16)	-0.57	-1.30	0.16	*	81.56 – 82.51	NA
Total Protein	22.17 (0.96) (21.53 – 22.74)	22.33 0.88) (21.82 -23.23)	-0.16	-2.78	2.46	NS	21.57 – 23.66	22.45 – 30.90
Total Fat	2.69 (0.09) (2.61 – 2.73)	2.66 (0.11) (2.54 – 2.90)	0.03	-0.26	0.31	NS	2.44 – 2.88	NA
Fibre (% dw)								
Acid Detergent Fibre	23.75 (0.63) (23.48 – 23.93)	24.11 (0.62) (23.71 – 24.58)	-0.36	-2.14	1.42	NS	23.08 – 24.79	25.1 – 40.3
Neutral Detergent Fibre	50.62 (0.73) (48.93 – 52.51)	49.10 (0.92) (46.74 – 50.50)	1.52	-0.83	3.88	NS	41.05 – 46.93	46.1 – 63.8
Dietary Fibre	*16.28 (0.52)* (15.36 – 17.24)	*16.77 (0.53)* (16.06 – 17.12)	-0.49	-1.99	1.01	NS	14.68 – 15.21	NA
Minerals (% dw)								
Phosphorus	0.29 (0.02) (0.28 – 0.30)	0.30 (0.02) (0.29 – 0.31)	-0.01	-0.07	0.06	NS	0.27 – 0.30	0.35
Calcium	0.35 (0.01) (0.32 – 0.37)	0.38 (0.01) (0.37 – 0.39)	-0.03	-0.06	0	*	0.33 – 0.37	0.24

SE: standard error of the mean; Sig: statistical significance; NS: no significant difference; *: statistically different ($p<0.05$); dw: dry weight; fw: fresh weight; [1]: ref. Obert, 2004; *Italics*: indicate value out of range.

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Table 23. Summary of forage nutrients for wheat event IND-ØØ412-7 vs. Parental control line Cadenza. Combined sites with or without glufosinate

Component (% dwt)	IND-ØØ412-7 Mean (SE)[d] Range	IND-ØØ412-7+G Mean (SE) Range	Cadenza Mean (SE) Range	Commercial Reference Range	Literature Range[e]
Proximates (% dwt) [a]					
Ash	14.62 (0.37) * (12.89 - 17.08)	14.50 (0.29) * (13.23 - 16.07)	15.61 (0.29) (14.34 - 17.28)	10.68 –16.68	NA
Carbohydrates [c]	37.9 (1.4) (29.1 - 46.2)	38.6 (1.3) (28.3 - 44.9)	37.4 (0.9) (31.7 - 43.0)	31.4 – 47.6	NA
Moisture (% fwt) [b]	9.51 (0.23) (8.54 - 10.95)	9.57 (0.29) (7.46 - 11.34)	9.22 (0.32) (7.64 - 11.76)	8.36 - 11.41	NA
Protein	*16.6 (0.5)* (14.1 - 20.5)	*17.5 (0.6)* (14.5 - 20.9)	*17.6 (0.4)* (15.6 - 19.5)	12.7 – 16.1	22.45 – 30.90
Total Fat	3.4 (0.1) (2.9 - 4.0)	3.3 (0.1) (2.7 - 3.9)	3.3 (0.2) (2.0 - 3.8)	2.9 – 4.0	NA
Fibre (% dwt)					
Acid Detergent fibre	31.3 (0.8) (26.3 - 35.2)	31.6 (0.6) (28.4 - 35.3)	30.4 (0.8) (26.4 - 35.0)	28.5 – 33.2	25.1 – 40.3
Neutral Detergent Fibre	51.9 (0.9) (47.1 - 56.4)	52.8 (1.0) (45.6 - 57.3)	51.1 (1.1) (46.3 - 57.0)	50.6 – 57.4	46.1 – 63.8
Dietary fibre	27.4 (1.1) (19.3 - 34.7)	26.1 (1.0) (21.0 - 32.4)	26.1 (0.8) (20.4 - 29.5)	21.1 – 34.2	NA
Minerals (mg/kg dwt)					
Calcium	0.33 (0.01) (0.29 - 0.40)	0.34 (0.02) (0.26 - 0.45)	0.33 (0.01) (0.26 - 0.37)	0.21 – 0.36	0.35
Phosphorus	0.46 (0.02) (0.32 - 0.59)	0.46 (0.02) (0.33 - 0.55)	*0.48 (0.01)* (0.39 - 0.55)	0.27 – 0.47	0.24

[a] dwt = dry weight; [b] fwt = fresh weight; [c] Determined by calculation; [d] SE = standard error of the mean; [e] Ref. Orbert, 2004; +G: with glufosinate; NA = not available; *Italics* indicates values out of range.

Table 24. Summary of seed anti-nutrients for wheat event IND-ØØ412-7 vs. Parental control line Cadenza. Combined-sites analysis with or without glufosinate

Component (% dwt) [a]	IND-ØØ412-7 Mean (SE)[b] Range	IND-ØØ412-7+G Mean (SE) Range	Cadenza Mean (SE) Range	Commercial Reference Range	Literature Range
Phytic acid	1.4 (0.1) (0.7 - 2.0)	1.4 (0.1) (0.8 - 2.2)	1.5 (0.1 (0.9 - 2.2)	1.1 – 2.5	0.49 – 0.93[c]
Gliadin	6.4 (0.2) (5.6 - 7.1)	6.4 (0.1) (5.5 - 6.9)	6.7 (0.1) (6.1 - 7.5)	6.1 – 7.1	3.9 – 9.1[d]

<u>**Conclusions from compositional analysis**</u>

In summary, the nutrient and anti-nutrient contents in grain and forage from the wheat event IND-ØØ412-7 were found to be equivalent to those measured in the parental non-transgenic control line, similar to the levels displayed by the commercial wheat reference varieties planted in the same locations, and comparable

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to the values reported in the literature. This equivalence was also shown to be not affected by glufosinate herbicide treatment. These results confirm that the transgenic event IND-ØØ412-7 is compositionally equivalent to conventional wheat and that this equivalence is insensitive to the treatment with glufosinate herbicide treatment.

C. Information related to the nutritional impact of the genetically modified food

Wheat has a long history of safe use. Global production in 2029[4] was more than 766 million tonnes. Most was consumed directly by humans and the remaining fed to animals.

The wheat event IND-ØØ412-7 in this submission has been transformed with gene cassettes designed to express the stress tolerance gene *HaHB4* and the *bar* gene to produce the PAT protein for herbicide tolerance. The introduction of the genetic modification had no nutritional impact on the wheat. This is supported by the fact that:

- Molecular characterisation demonstrated stability of the inserts during numerous generations
- The HAHB4 protein is part of an HD-Zip 1 family found across all plants, with a history of safe consumption and no significant homology to known allergens and toxins; and
- Compositional analysis did not indicate biologically significant changes to the levels of nutrients in events compared to their conventional counterparts. Event composition is within the normal variation of wheat varieties and is substantially equivalent to conventional wheat.

The difference between the HB4 wheat and the untransformed control, relate to low levels of the newly expressed HAHB4 protein and the PAT protein. However, the expression of these two new proteins did not alter the compositional profile. Thus, food products derived from HB4 wheat are anticipated to be nutritionally equivalent to food products derived from other commercially available wheat, except that HB4 wheat is tolerant to environmental stress and has herbicide tolerance.

D. Other Information

Where a biotech food has been shown to be compositionally equivalent to conventional varieties, the evidence to date indicates that feeding studies will add little to the safety assessment and generally are not warranted (see e.g. Bartholomaeus et al., 2013; Herman and Ekmay, 2014; OECD, 2003). However, a nutritional assessment was undertaken to compare HB4 wheat with the near isogenic parental line on broiler chicken performance (see **Supplement Report D Nutritional Study**). No significant differences were observed between wheat event IND-ØØ412-7 and its near-isogenic parental variety Cadenza, regarding zootechnic parameters and carcass characteristics.

The new polypeptide produced by the inserts in wheat event IND-ØØ412-7 have been well characterised and are prevalent in the food chain. The proteins are non-toxic and occurs at very low levels in the transformed plant. Its safety is supported by a weight-of-evidence that indicates safety for human consumption. Considering the compositional equivalence between the wheat event and its conventional variety, and the lack of any observed phenotypic characteristics indicative of unintended effects arising from the genetic modification process, there was no plausible risk hypothesis that would indicate the need for animal feeding studies.

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Appendix 1.

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Figure 23. Sequence of the long insert
Nucleotides corresponding to specific elements of the insert are highlighted in different colours. Wheat genome is indicated in green, *HaHB4* in red and *bar* in light blue. Orange and violet indicate *bla* and *gus*, respectively.

INFORMATION IN THIS FIGURE IS COMMERCIALLY SENSITIVE

Figure 24. Sequence of the short insert

Nucleotides corresponding to specific elements of the insert are highlighted in different colours. Wheat genome is indicated in green, *HaHB4* in red and *bar* in light blue. Orange and violet indicate *bla* and *gus*, respectively. ^: symbol introduced to separate two contiguous copies of *bla* to allow visualisation.

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Appendix 2.

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Figure 25. Junction Point Sequences Supported by Illumina and PacBio Reads, and by PCR Amplification Products Sequenced by Sanger.
Nucleotide sequences corresponding to the junction points at both sides (a and b) of the long (JPL) and short (JPS) inserts are shown. Wheat sequences are highlighted in green, the first 100 bp of the insert in yellow (for *bla*) or blue (for *prUbi-1*) and the backbone in grey. Numbers represent absolute position on the insert sequences.

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