FOOD STANDARDS
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Te Mana Kounga Kai – Ahitereiria me Aotearoa

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Supporting document 1

Safety assessment – **Application A1232**

Food derived from drought-tolerant and herbicide-tolerant wheat line IND-00412-7

Executive summary

Background

Application A1232 seeks approval for the sale and use of food derived from genetically modified (GM) wheat line IND-00412-7 that has tolerance to drought and the herbicide glufosinate.

Drought tolerance is conferred by the expression of the novel transcription factor HaHB4, encoded by the *HaHB4* gene from sunflower. This novel transcription factor regulates gene transcription in IND-00412-7 wheat in response to environmental stressors, such as drought. The HaHB4 protein has not previously been assessed by FSANZ.

Tolerance to glufosinate is achieved through expression of the enzyme phosphinothricin acetyltransferase (PAT), encoded by the *bar* gene from the soil bacterium *Streptomyces hygroscopicus.* The PAT protein has previously been assessed by FSANZ.

This safety assessment addresses food safety and nutritional issues associated with the GM food. It therefore does not address:

- risks related to the environmental release of GM plants used in food production
- risks to animals that may consume feed derived from GM plants
- the safety of food derived from the non-GM (conventional) plant.

History of use

Wheat is grown worldwide as a commercial food crop, but also to supplement animal feed. It has a long history of safe use in the food supply, dating back thousands of years. Wheat is typically milled into flour and used to make a variety of food products including bread, pasta, biscuits and other baked goods.

Molecular characterisation

The genes encoding HaHB4 (*HaHB4*) and PAT (*bar*) were introduced into wheat line IND-00412-7 via particle bombardment. The transformation also introduced elements of the GUS reporter system, which was used to monitor plant transformation efficacy, as well as the *bla*

antibiotic resistance gene used to select plasmid carrying bacteria.

Detailed molecular analyses of wheat line IND-00412-7 indicate that multiple copies of the *HaHB4*, *bar, gus* and *bla* genes are present at two insertion sites at one locus on the same chromosome. Genes were either intact or incomplete, with even fewer functional copies. The sequencing data confirmed a single fully functional *HaHB4* and two functional *bar* expression cassettes exist in the genome of IND-00412-7. Due to the lack of fully intact or eukaryotic regulatory elements, the *gus* and *bla* genes are unlikely to be expressed.

The introduced genetic elements were shown by molecular techniques and phenotypic analyses to be stably inherited across multiple generations. The pattern of inheritance supports the conclusion that the introduced traits occur within a single locus in the IND-00412-7 genome and are inherited in accordance with Mendelian principles.

Characterisation and safety assessment of new substances

Wheat line IND-00412-7 expresses the novel protein HaHB4. There is a history of human exposure to this protein though the consumption of sunflower seed, and homologous proteins found in the plant kingdom and commonly consumed food. As a transcription factor, it is expressed at very low levels in plants. Bioinformatic studies confirmed a lack of any significant amino acid sequence similarity between HaHB4 and known protein toxins or allergens. Laboratory studies also demonstrated HaHB4 is susceptible to the digestive enzyme pepsin and would be thoroughly degraded before it could be absorbed during passage through the gastrointestinal tract. Additionally, *in silico* analysis of HaHB4 for glycosylation sequences indicated that this post-translational modification of HaHB4 in wheat is unlikely. Taken together, the evidence supports the conclusion that HaHB4 is not toxic or allergenic in humans.

PAT is a newly expressed protein present in IND-00412-7. It is expressed in various tissues, with relatively low levels in grain. A range of characterisation analyses confirmed the identity of PAT in IND-00412-7. The safety of this proteins has been assessed by FSANZ in numerous previous applications. Updated bioinformatic analyses undertaken for this application confirmed the expressed protein is unlikely to be allergenic or toxic.

Herbicide metabolites

For PAT, the metabolic profiles resulting from the novel protein/herbicide interaction have been established through a significant history of use. The glufosinate-tolerance trait is present in lines in close to thirty previous applications to FSANZ. There are no concerns that the spraying of IND-00412-7 with glufosinate would result in the production of metabolites that are not also produced in non-GM crops sprayed with the same herbicide and already used in the food supply.

Compositional analyses

Detailed compositional analyses were performed on IND-00412-7. Analytes measured were proximates (ash, carbohydrates, moisture, protein, fat), fibre, amino acids, vitamins, minerals, fatty acids and anti-nutrients. Statistically significant differences were found between grain from IND-00412-7 and the control for 3 of the 43 analytes evaluated, however differences were within the range of existing commercial non-GM wheat cultivars. Overall, the compositional data support the conclusion that there are no biologically significant differences in the levels of key constituents in grain from IND-00412-7 compared to non-GM wheat cultivars available on the market.

Conclusion

No potential public health and safety concerns have been identified in the assessment of drought-tolerant and herbicide-tolerant wheat line IND-00412-7. On the basis of the data provided in the present application, and other available information, food derived from IND-00412-7 is considered to be as safe for human consumption as food derived from non-GM wheat cultivars.

TABLE OF CONTENTS

Index of Figures

Index of Tables

List of Abbreviations

Abbreviation	Description
bp	base pair
BSA	bovine serum albumin
DNA	deoxyribonucleic acid
DArT	diversity arrays technology
dw	dry weight
ELISA	enzyme-linked immunosorbent assay
fw	fresh weight
FSANZ	Food Standards Australia New Zealand
g	gram
GM	Genetically modified
kDa	kilodalton
min	minutes
LC-MS	liquid chromatography mass spectrometry
MT	Million tons
MALDI-TOF	Matrix-assisted laser desorption/ionization
NCBI	National Centre for Biotechnology Information
OECD	Organization for Economic Co-operation and Development
OGTR	Office of the Gene Technology Regulator
ORF	open reading frame
PAT	phosphinothricin acetyltransferase
PCR	polymerase chain reaction
RNA	ribonucleic acid

Abbreviation	Description
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SE	standard error
U	unit
µg	microgram

1 Introduction

FSANZ has received an application from Trigall Genetics to vary Schedule 26 in the *Australia New Zealand Food Standards Code* (the Code). The variation is to include food derived from the genetically modified (GM) wheat line IND-00412-7, with the Organisation for Economic Cooperation and Development (OECD) Unique Identifier IND-ØØ412-7. This wheat line is tolerant to drought and the herbicide glufosinate.

Drought tolerance is achieved through expression of a sunflower transcription factor HaHB4 that drives expression of abiotic stress response genes. The response increases the plant's tolerance to conditions of water scarcity and other environmental stresses such as salinity and grazing damage. Tolerance to the herbicide glufosinate is achieved by the expression of phosphinothricin acetyltransferase (PAT) enzyme. PAT is encoded by the *bar* gene, which is derived from the bacterium *Streptomyces hygroscopicus*. Unlike the PAT protein, the HaHB4 protein has not been assessed previously by FSANZ.

2 History of use

2.1 Host organism

The host organism is wheat (*Triticum aestivum L*) and the parental variety used for the genetic modification is Cadenza. Cadenza is a bread wheat variety that is grown in the spring. The Cadenza host organism was used as the conventional control for the purposes of comparative assessment with IND-00412-7.

Wheat has a long history of cultivation and human consumption (Balfourier et al., 2019; Shiferaw et al., 2013). The commodity is a major contributor to daily dietary calories and protein, and is of worldwide economic importance. For more detailed information, please refer to reports published by the OECD (2003a), the Grains Research and Development Corporation (GRDC 2019) and the Office of the Gene Technology Regulator (OGTR 2021).

Wheat is grown worldwide as a commercial food crop but also to supplement animal feed. It is one of the world's most dominant cereal crops and in 2020/21 worldwide production was 775 MT[1] (USDA 2021). China, the European Union and India are the largest producers and in 2020/21 production reached 134, 126 and 108 MT, respectively. Wheat is grown in Australia and in 2020/21 production was approximately 33 MT. Wheat is not a major crop in New Zealand. In 2019 production was approximately 0.398 MT (FAOSTAT 2019).

A large amount of Australian wheat grain is exported. In 2019 exports amounted to 9.6 MT (FAOSTAT 2019). In the same year, 0.489 MT of wheat grain and 0.029 MT of wheat flour was imported. New Zealand wheat grain export in 2019 was 0.0001 MT. In the same year New Zealand imported 0.430 MT of wheat grain and 0.02 MT of wheat flour. These imports are used in processed foods.

Wheat has a long history of safe use as food for human consumption (Shewry and Hey, 2015; OECD 2003a). Typically, wheat is milled into flour and used to make a variety of food products. These include bread, pasta, biscuits and other baked goods.

[1] million tons

2.2 Donor organisms

2.2.1 *Helianthus annuus*

The *HaHB4* DNA sequence encoding the HaHB4 protein is derived from *Helianthus annuus*, also known as the common sunflower. The sunflower is native to North America and has been grown as a food crop for thousands of years (Lentz et al., 2008). Sunflower seeds are either eaten whole, milled for flour or meal to make bread and other baked goods, or the oil is extracted and used for cooking (Adeleke and Babalola, 2020). Hence, the seeds have a history of safe human consumption.

2.2.2 *Streptomyces hygroscopicus*

The *bar* gene encodes the PAT protein and is derived from *Streptomyces hygroscopicus*, a non-pathogenic gram-positive spore-forming bacterium found in soil. FSANZ has previously assessed and approved 9 applications containing this gene sequence. At the international level, the *bar* gene has been used to confer tolerance to glufosinate ammonium herbicides in food producing crops for over two decades (OECD 2006; CERA 2011).

2.2.3 Other organisms

Genetic elements from several other organisms have been used in the genetic modification of IND-00412-7 (refer to Table 1 and 2). These genetic elements are either:

- non-coding sequences that are used to regulate the expression of *HaHB4* and *bar* genes
- coding and non-coding sequences from the transformation plasmids and a reporter system.

The potential of the latter sequences resulting in unintended protein expression is addressed in Section 3.4.1 and 3.4.3.

3 Molecular characterisation

Molecular characterisation is necessary to provide an understanding of the genetic material introduced into the host genome and helps to frame the subsequent parts of the safety assessment. The molecular characterisation addresses three main aspects:

- the transformation method together with a detailed description of the DNA sequences introduced to the host genome
- a characterisation of the inserted DNA, including any rearrangements that may have occurred as a consequence of the transformation
- the genetic stability of the inserted DNA and any accompanying expressed traits.

3.1 Transformation method

In order to create the IND-00412-7 wheat line, the wheat variety Cadenza was transformed using the pIND4-HB4 and pIND4-Bar plasmids (Figures 1 and 2). The methodology is outlined in the flowchart in Appendix 1 and summarised below.

The transformation method involved particle bombardment of immature Candeza wheat embryos with plasmid DNA. In addition to pIND4-HB4 and pIND4-Bar, a third plasmid was used in the transformation process. This plasmid contained the GUS reporter system, which includes the *gus* gene encoding a β-glucuronidase. The β-glucuronidase enzyme converts a colourless substrate into a coloured product. The GUS reporter system is commonly used to

determine the effectiveness of the transformation process (Jefferson 1989; Hull and Devic, 1995; Guivarc'h et al., 1996).

Following transformation, embryos were cultured in the dark for three weeks in medium that promotes callus formation. Embryonic calluses were then cultured in regeneration medium under light until shoots developed. At this stage, plantlets were placed in selective media containing glufosinate. Glufosinate inhibits the grown of untransformed plant cells. Regenerated plants were transferred to soil and were screened for the presence of *HaHB4* and *bar* genes using standard molecular biology techniques. Following the evaluation of insert integrity, gene expression, phenotypic characteristics and agronomic performance, wheat line IND-00412-7 was selected.

3.2 Detailed description of inserted DNA

The transformation process that generated the IND-00412-7 wheat line makes use of the pIND4-HB4 and pIND4-Bar plasmids, one containing the *HaHB4* expression cassette and the other containing the *bar* expression cassette. Each plasmid is described below.

3.2.1 pIND4-HB4

The pIND4-HB4 plasmid contains the *HaHB4* expression cassette. A plasmid map showing the location of each of the genetic elements is in Figure 1 and a description of the genetic elements contained within the plasmid is in Table 1

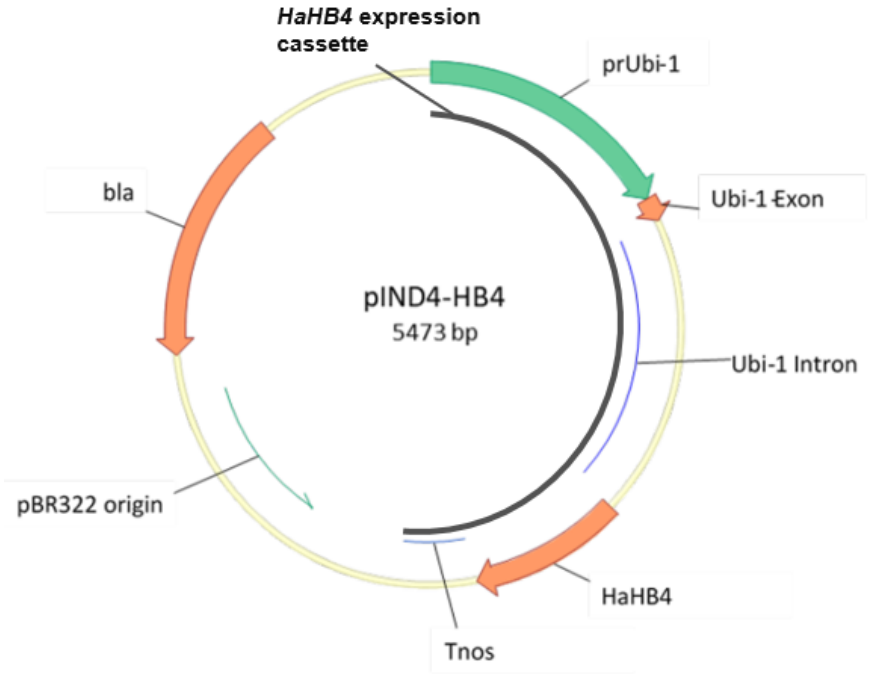


Figure 1: Plasmid map of pIND4-HB4. The genetic elements that comprise the HaHB4 expression cassette is highlighted using the grey bar.

HaHB4 expression cassette

The *HaHB4* coding sequence is from *Helianthus annuus*. Expression of *HaHB4* is under the control of the promoter, 5' untranslated exon and first intron of the *Ubi-1* (ubiquitin) gene from *Zea mays* (corn). Together, these genetic elements are highly active in monocotyledonous plants (such as wheat) and provide a strong enhancing effect on the expression of transgenes in cereals (Vasil et al., 1993; Christensen et al., 1992). Downstream of the coding

sequence is the Tnos genetic element, which is a polyadenylation sequence of the nopaline synthase gene from *Agrobacterium tumefaciens*. This sequence is required for the termination of transcription.

Plasmid backbone

The DNA contained within the backbone region of the plasmid is required for preparing the plasmid and passaging through standard laboratory *Escherichia coli*. The pBR322 genetic element is a bacterial origin of replication and the *bla* gene is an ampicillin resistance gene from *E. coli*. Ampicillin resistance is used as a selectable marker for plasmid containing bacteria. The presence of the *bla* gene in wheat line IND-00412-7 is covered in Section 3.4.2 and the safety of any potentially expressed *bla* protein is covered in Section 3.4.3.

Table 1: Genetic elements present in the pIND4-HB4 plasmid

Genetic element	Position	Size (bp)	Source	Description, Function & Reference
***HaHB4* expression cassette**				
Ubiquitin (Ubi-1) promoter	1–898	898	*Zea mays*	Promoter sequence of the *Ubi-1* gene encoding ubiquitin that directs transcription in plant cells (Christensen and Quail, 1996; Christensen et al., 1992)
Ubiquitin (Ubi-1) Exon	899–981	83	*Zea mays*	5' untranslated exon of the Ubi-1 gene encoding ubiquitin that facilitates transcription in plant cells (Christensen and Quail, 1996; Christensen et al., 1992)
Intervening sequence	982–995	14	pUC plasmid	Sequence used for cloning
Ubiquitin (Ubi-1) Intron	996–2,005	1,010	*Zea mays*	First intron of the Ubi-1 gene encoding ubiquitin that facilitates transcription in plant cells (Christensen and Quail, 1996; Christensen et al., 1992)
Intervening sequence	2,006–2031	26	pUC plasmid	Sequence used for cloning
HaHB4 gene	2,032–2,565	534	*Helianthus annus*	Coding sequence of the *HaHB4* gene for the HaHB4 transcription factor that drives the expression of abiotic stress response genes (Manavella et al., 2006; Dezar et al., 2005)
Intervening sequence	2,566–2,581	16	pUC plasmid	Sequence used for cloning
Tnos	2,582–2,834	253	*Agrobacterium tumefaciens*	Polyadenylation signal of the nopaline synthase gene that terminates transcription (Depicker et al., 1982)
Intervening sequence	2,835–3,239	405	pUC plasmid	Sequence used for cloning
pBR322 origin of replication	3,240–3,859	620	Synthetic pBR322 plasmid	Plasmid origin of replication (Yanisch-Perron et al., 1985)
Intervening sequence	3,860–4,013	154	pUC plasmid	Sequence used for cloning
Bla gene	4,014–4,874	861	*Escherichia coli*	β-lactamase encoding for ampicillin resistance that allows the selection of plasmid carrying bacteria (Briñas et al., 2002; Sutcliffe 1978)
Intervening sequence	4,875–5473	599	pUC plasmid	Sequence used for cloning

3.2.2 pIND4-Bar

The pIND4-Bar plasmid contains the bar expression cassette. A plasmid map showing the location of each of the genetic elements is in Figure 2 and a description of the genetic elements contained within the plasmid is in Table 2.

bar expression cassette

The *bar* coding sequence is from *E. coli*. Expression of *bar* is under the control of the same promotor and terminator elements as the *HaHB4* expression cassette. The same parental plasmid was used in the construction of both plasmids.

Plasmid backbone

The genetic elements contained within the backbone of pIND4-Bar are identical to the pIND4-HB4 plasmid.

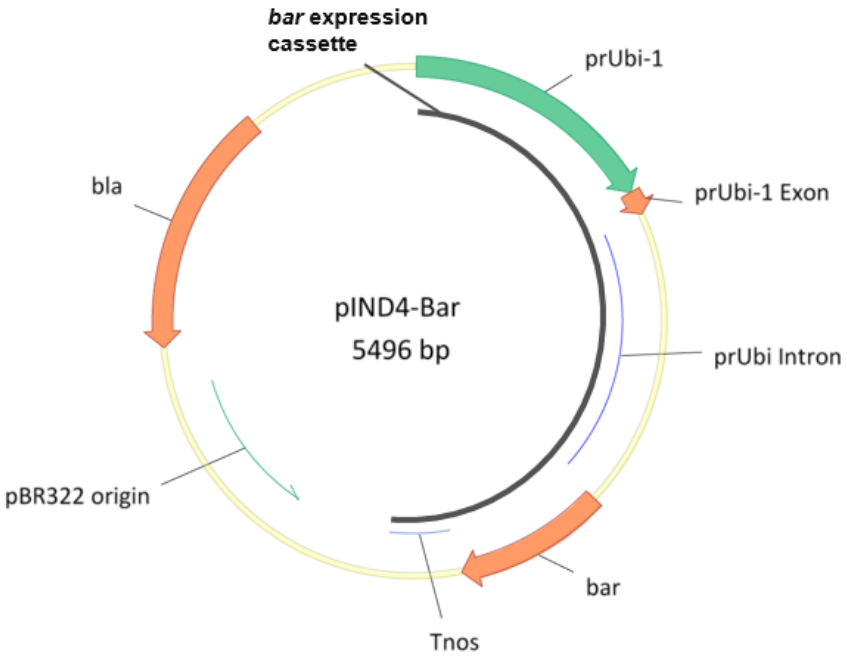


Figure 2: Plasmid map of pIND4-Bar. The genetic elements that comprise the Bar expression cassette is highlighted using the grey bar.

Table 2: Genetic elements present in the pIND4-Bar plasmid

Genetic element	Position	Size (bp)	Source	Description, Function & Reference
***bar* expression cassette**				
Ubiquitin (Ubi-1) promoter	1–898	898	*Zea mays*	Promoter sequence of the *Ubi-1* gene encoding ubiquitin that directs transcription in plant cells (Christensen and Quail, 1996; Christensen et al., 1992)
Ubiquitin (Ubi-1) Exon	899–981	83	*Zea mays*	5' untranslated exon of the Ubi-1 gene encoding ubiquitin that facilitates transcription in plant cells (Christensen and Quail, 1996; Christensen et al., 1992)
Intervening sequence	982–995	14	pUC plasmid	Sequence used for cloning
Ubiquitin (Ubi-1) Intron	996–2,005	1,010	*Zea mays*	First intron of the Ubi-1 gene encoding ubiquitin that facilitates transcription in plant cells (Christensen and Quail, 1996; Christensen et al., 1992)
Intervening sequence	2,006–2041	36	pUC plasmid	Sequence used for cloning

Genetic element	Position	Size (bp)	Source	Description, Function & Reference
bar gene	2,042–2,590	549	*Streptomyces hygroscpicus*	Coding sequence of the *bar* gene for the PAT protein that provides tolerance to glufosinate (Thompson et al., 1987; White et al., 1990)
Intervening sequence	2,591–2,607	17	pUC plasmid	Sequence used for cloning
Tnos	2,608–2,860	253	*Agrobacterium tumefaciens*	Polyadenylation signal of the nopaline synthase gene that terminates transcription (Depicker et al., 1982)
Intervening sequence	2,861–3,263	403	pUC plasmid	Sequence used for cloning
pBR322 origin of replication	3,264–3,883	620	Synthetic pBR322 plasmid	Plasmid origin of replication (Yanisch-Perron et al., 1985)
Intervening sequence	3,884–4,037	154	pUC plasmid	Sequence used for cloning
Bla gene	4,038–4,898	861	*Escherichia coli*	β-lactamase encoding for ampicillin resistance that allows the selection of plasmid carrying bacteria (Briñas et al., 2002; Sutcliffe 1978)
Intervening sequence	4,899–5496	599	pUC plasmid	Sequence used for DNA cloning

3.2.3 Other sequences

Incomplete sequences of the prGBl-1 promoter, *gus* gene, and 35S terminator sequence has been inserted into the IND-00412-7 genome (described further in Section 3.4.1). The *gus* coding sequence is from the *uidA* gene in *E. coli* (Jefferson et al., 1987). Expression of *gus* is under the control of the prGbl-1 promoter from *Triticum aestivum L.* (wheat) and the 35S terminator sequence from the cauliflower mosaic virus (CaMV) (Sanfaçon et al., 1991). The potential for these sequences to result in the expression of *gus* in IND-00412-7 is addressed in Section 3.4.1.

3.3 Development of the wheat line from original transformant

A breeding pedigree for the development of IND-00412-7 and the various generations used in the characterisation of IND-00412-7 is given in Appendix 2. From the transformed T0 plant, several rounds of self-pollination and crosses occurred in order to produce specific generations of plants that were used in characterisation and analysis of IND-00412-7, as indicated in Table 3.

Conventional breeding programs will be used to cross the original event (IND-00412-7) and its progeny with elite wheat varieties, resulting in commercial wheat varieties for food use.

Table 3: IND-00412-7 generations used for various analyses

Analysis	Section	Generation(s) used	Comparators
Molecular characterisation of insert(s)	Section 3.4.1	T7 F2[2]	non-GM Cadenza
Genetic stability	Section 3.4.2	T5, T6, T7, T7 F2	N/A
Phenotypic stability	Section 3.4.2	Various e.g. T0, T4, T8, T9, T10	non-GM Cadenza, parental IND-00412-7 and FD38
HaHB4 expression	Section 4.1.1	T7, T10	non-GM Cadenza

[2] IND-00412-7 x Baguette 17

Analysis	Section	Generation(s) used	Comparators
PAT expression	Section 4.2.1	T8	non-GM Cadenza
Compositional analysis	Section 5	T9	non-GM Cadenza

3.4 Characterisation of the inserted DNA and site(s) of insertion

A range of analyses were undertaken to characterise the genetic modification in IND-00412-7. These analyses focused on the nature and stability of the insertion and whether any unintended re-arrangements or products may have occurred as a consequence of the transformation procedure.

The use of particle bombardment as a transformation method can at times result in a complex integration structure (Altpeter et al., 2005; Kohli et al., 2003). This includes the insertion of multiple intact copies, as well as rearranged and/or truncated genes and fragments. Considering the transformation method involved whole plasmids, it is expected that vector backbone fragments at the insertion locus will be found in wheat line IND-00412-7.

The integration structure and stability of the IND-00412-7 event is examined below.

3.4.1 Analysis of the integrated novel DNA

Southern blot analysis was used to analyse the insertion site(s) and determine copy number. Genomic DNA from IND-00412-7 was digested with restriction enzymes (*Hin*d III, *Bam*HI or *Ase*I) and hybridised with DIG-labelled probes for either *HaHB4* or *bar*. The non-transformed host cultivar Cadenza was used as a negative control. A previously characterised soybean line (HB4 soybean), containing *HaHB4* and *bar*, was used as the positive control. As expected, no hybridisation was observed in the Cadenza samples while the data for IND-00412-7 revealed a complex pattern of transgene insertion with multiple inserts for both genes.

To further resolve the complex integration structure, genome sequencing was required. As the host cultivar Cadenza is a hexaploid[3], a standard whole genome sequencing approach is not optimal (Zimin et al., 2017). To address this limitation, diversity arrays technology (DArT) was used to localise the *HaHB4* and *bar* insert to a specific chromosome in the wheat genome (Jaccoud et al., 2001; Akbari et al., 2006). Following the identification of the chromosome carrying the *HaHB4* and *bar* gene(s), a flow cytometry sorting method was used to isolate the specific chromosome where integration had occurred. A combination of Illumina (short reads) and PacBio (long reads) sequencing was then performed on the isolated chromosomal material (Zimin et al., 2017).

The sequencing data showed there were four junction points, indicating two insertion sites. The sites of the insertion were identified and the flanking genomic DNA from the host was provided to FSANZ. The integration event in IND-00412-7 consists of two inserts in one locus on the same chromosome. The data shows the inserts have not disrupted any known endogenous genes.

The sequencing data confirmed a single fully functional *HaHB4* and two functional *bar* expression cassettes exist in the genome of IND-00412-7 (Table 4). The presence of several copies of truncated expression cassette sequences, rearranged sequences and plasmid

[3] six copies of each chromosome

backbone components were found. The presence of rearranged and truncated DNA is not unexpected from particle bombardment (Pawlowski and Somers 1996; Kohli et al., 1998; Mehlo et al., 2000) and the mere presence of the fragmented DNA does not raise potential safety concerns. The potential for this DNA to lead to protein expression is addressed in Section 3.4.3. The data from the sequencing were consistent with the Southern blotting results.

Table 4: Gene copy number in wheat line IND-00412-7

Gene	Gene copy number		Functional copies[1]
	Intact	Incomplete	
HaHB4	2	1	1
bar	7	1	2
bla	12	7	Unlikely to be expressed
gus	0	4	Unlikely to be expressed

1. Genes with complete/functional regulatory elements

The sequencing data confirmed the presence of intact copies of *bla* (Table 4). The *bla* gene is derived from *E. Coli* and is present on the plasmid backbone of both pIND4-HB4 and pIND4-Bar (Section 3.2). The gene is under the control of a bacterial promoter and as a result, is unlikely to be expressed in plants. The safety of any potentially expressed *bla* protein is covered in Section 3.4.3.

The sequence analysis also identified additional sequences from a *gus* expression cassette that are inserted in the same chromosome (Table 4; Section 3.2.3). This includes incomplete copies of the prGbl-1 promoter, *gus* gene and 35S terminator sequence. Typically such sequences would be eliminated through segregation post-transformation however, in this instance, they are co-localised with the *HaHB4* and *bar* gene inserts which means they cannot be segregated away. While there are multiple DNA inserts derived from the *gus* expression cassette, the *gus* sequences themselves are truncated and therefore non-functional, nor are they likely to be expressed due to the absence of intact regulatory elements. The presence of these additional DNA sequences do not raise any safety concerns.

While the characterisation has focussed on the chromosome into which the *HaHB4* and *bar* genes were inserted, the likelihood of insertions in other parts of the genome is considered to be very low. DArT has already confirmed a single locus of insertion for the *HaHB4* gene and *bar* genes, and sequence analysis also demonstrated the presence of additional sequences at the same locus. The use of particle bombardment to introduce multiple expression cassettes typically results in insertion of DNA sequences at a single locus in the genome (Altpeter et al., 2005).

3.4.2 Stability of the genetic changes in wheat line IND-00412-7

The concept of stability encompasses both the genetic and phenotypic stability of the introduced trait over a number of generations. Genetic stability refers to maintenance of the modification (as produced in the initial transformation events) over successive generations. Phenotypic stability refers to the expressed trait remaining unchanged over successive generations.

Mendelian inheritance

Since the integrating event in IND-00412-7 consists of two inserts in one locus, the genetic material within it would be expected to be inherited according to Mendelian principles. Chi-square (X^2) analysis was undertaken in progeny of T7 F2 plants and across several generations to confirm the segregation and stability of the inserted DNA.

A segregation analysis was performed on progeny derived from a cross between IND-00412-7 at T7 and a non-GM commercial cultivar Baguette 17. The resulting F1 progeny were self-pollinated and 349 F2 seeds were analysed by endpoint PCR for the presence of the complete coding sequences of *HaHB4* and *bar*. The expected ratio of progeny containing both *HaHB4* and *bar* to progeny not containing both traits (null) is 3:1. The observed ratio presented in Table 5 matches the expected results, indicating the introduced traits follow expected Mendelian inheritance rules.

Table 5: Segregation results for both *HaHB4* and *bar* in IND-00412-7 x Baguette 17 at F2

	Presence of both *HaHB4* and *bar*	
	Observed	**Expected**
Present	259	261.75
Null	90	87.25
Total plants	349	
X^2	0.11	
p	0.74	

Further analysis was performed on a subset of the F2 seeds (92 seeds) using PCR. The analysis examined the four junction regions, the *bla* gene, and truncated *HaHB4* and *bar*. The expected ratio of presence to null for each target was 3:1. The observed results matched the expected results for all genetic elements analysed. These data provide further support of Mendelian inheritance and confirmed there is genetic stability of the novel DNA insertions across multiple cross-breeding steps and generations.

In addition to the segregation studies above, the applicant provided further data on the detection of inserted DNA across generations T5, T6 and T7 (Appendix 2). Using the same primers as the segregation studies, the presence of the *HaHB4*, *bar* and *bla* genes and junction regions were analysed. All generations were shown to contain the inserted genetic elements, further confirming the stability of the insertion locus across multiple generations.

Expressed phenotype over several generations

The applicant has provided RNA expression data showing that both *HaHB4* and *bar* genes are expressed in multiple lines developed from T4 and T10 generations (Appendix 2). At the protein level, IND-00412-7 displays glufosinate tolerance or expresses the PAT protein at multiple generations, including T0, T8, T9. Due to the intractable levels of HaHB4, protein levels are difficult to quantify (Section 4.1.1). However, IND-00412-7 displays drought tolerance across multiple growing seasons and regions (Gonzalez et al., 2019). Together this indicates the drought and glufosinate tolerance phenotypes in IND-00412-7 are stable over several generations.

3.4.3 Open reading frame (ORF) analysis

The applicant has provided the results of *in silico* analyses to identify whether any novel ORFs had been created in IND-00412-7 as a result of the DNA insertion. Sequences spanning both inserts in IND-00412-7 and the 5' and 3' flanking genomic DNA (200 base pairs) were translated using from start-to-stop codon in all six reading frames using a BioPython script. If small putative proteins were contained within a larger sequence, the entire larger sequence was included in the ORF count and subsequent *in silico* analysis.

In addition to the HaHB4 and PAT novel proteins, a total of 67 ORFs that corresponded to putative peptides of greater than 100 amino acids were identified. The 67 putative peptides

were screened using the NCBI protein BLAST search tool[4]. The NCBI search showed 22 putative peptides did not align significantly (E score $<10^{-5}$) to any protein in this database. 10 had significant homologies with the newly expressed proteins (HaHB4 and PAT), 18 are associated with known coding sequences (Bla, GUS) and 17 were similar to vectors and/or hypothetical proteins. Putative peptides were used as query sequences in homology searches for known allergens and toxins in established databases.

These analyses are theoretical only as there is no reason to expect that any of the identified ORFs would, in fact, be expressed.

3.4.3.1 Bioinformatic analysis for potential allergenicity

The 67 putative peptides were queried against known allergenic proteins listed in the Allergen Online database[5] (version 21). At the date of the search, there were 2,233 sequences in the allergen database. With an 80-mer sliding window, none of the putative peptides shared similarity ≥ 35% to any known allergen. Similar negative results were obtained using an 8-mer sliding window.

A conformational analysis of the putative peptides against known allergens was also performed, with the Structural Database of Allergenic Proteins[6]. This database groups 1526 allergens, 1312 protein sequences, 92 crystallographic structures, 458 three-dimensional models and 29 IgE epitopes. No similarity was identified for any of the 67 putative peptides, when queried against all allergens and food allergens.

3.4.3.2 Bioinformatic analysis for potential toxicity

Putative peptides were examined for the presence of any known toxins found in the Toxin and Toxin Target Database[7] and the Toxin-antitoxin database[8]. Significant homology was determined based on a E score of $<10^{-5}$ and was detected with PAT-associated peptides (considered in Section 4.2.3). No other significant homology was found with the putative peptides and known toxins.

3.4.4 Conclusion

The data provided by the applicant showed that two integration events have occurred at a single locus in the wheat genome. Multiple copies of the *HaHB4*, *bar, gus* and *bla* genes are present, either intact or incomplete, with even fewer functional copies. The sequencing data confirmed a single fully functional *HaHB4* and two functional *bar* expression cassettes exist in the genome of IND-00412-7. Due to the lack of fully intact or eukaryotic regulatory elements, the *gus* and *bla* genes are unlikely to expressed. The introduced DNA was shown to be stably inherited from one generation to the next. No new ORFs are created by the insertions that raise potential allergenicity or toxicity concerns.

4 Characterisation and safety assessment of novel substances

In considering the safety of novel proteins it is important to understand that a large and diverse range of proteins are ingested as part of the normal human diet without any adverse effects. Only a small number of dietary proteins have the potential to impair health, because

[4] blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastp&PAGE_TYPE=BlastSearch
[5] www.allergenonline.org
[6] fermi.utmb.edu/
[7] www.t3db.ca/
[8] bioinfo-mml.sjtu.edu.cn/TADB2/tools.html

of anti-nutrient properties or triggering of allergies in some consumers (Delaney et al., 2008). As proteins perform a wide variety of functions, different possible effects have to be considered during the safety assessment including potential toxic, anti-nutrient or allergenic effects.

To effectively identify any potential hazards, knowledge of the characteristics, concentration and localisation of all newly expressed proteins in the organism as well as a detailed understanding of their biochemical function and phenotypic effects is required. It is also important to determine if the newly expressed protein is expressed in the plant as expected, including whether any post-translational modifications have occurred.

No protein products from the *bla* and *gus* genes are expected in the wheat line IND-00412-7. The *bla* gene is under the control of a bacterial promoter and lacks regulatory sequences that would be recognised in plants (EFSA 2009; FSANZ 2001; FSANZ 2000). The *gus* gene is truncated and does not contain an intact promoter, therefore is not likely to be expressed. FSANZ has also considered the safety of *bla* and *gus* in previous assessments[9,10]. The presence of *bla* and *gus* DNA sequences in IND-00412-7 is not considered to be of significance or concern.

Two novel substances are expressed in IND-00412-7, HaHB4 and PAT, and are assessed below.

4.1 HaHB4

The *Helianthus annuus* homeobox 4 (HaHB4) protein is a transcription factor. It is involved in regulating gene transcription in response to environmental stressors such as drought (Gonzalez et al., 2019; Manavella et al., 2008; Dezar et al., 2005).

Wheat line IND-00412-7 expressing HaHB4 protein shows increased grain yield compared to its non-GM control (Gonzalez et al., 2019). The relative increase in grain yields in HaHB4 wheat is correlated to water use efficiency under conditions of water scarcity. The percentage benefit of grain yield was larger (16%) in water-deficient environments compared to non-stressed environments (3%). Differences in grain yield is reflected in an increased number of grain. This in turn is associated with an increased crop growth rate during the critical period for grain number determination, as well as positive trends in spikelet numbers per spike, tillers per plant, and fertile florets per plant.

HaHB4 is a member of the homeodomain-leucine zipper (HD-Zip) gene family. The gene family is unique to the plant kingdom and found in food crops with a history of safe use such as rice and wheat (Yue et al., 2018; Ariel et al., 2007). Homologous sequences to HaHB4 are found in commonly consumed food, such as artichoke, golden kiwifruit and citrus. Sunflower, the source of the gene encoding the HaHB4 protein, also has a long history of safe use as food (see Section 2.2.1). Hence there is a history of human exposure to this specific protein as well as proteins from the same gene family.

Compared to the sunflower HaHB4, the HaHB4 in IND-00412-7 shares 96.1% sequence similarity or seven amino acid differences (Figure 3). This includes a deletion of four amino acids at positions 7-10, and three amino acid substitutions: lysine to arginine at position 22, phenylalanine to leucine at position 159, and proline to leucine at position 175. These changes do not impact on the conserved domains of the HD-Zip family. The proline to leucine substitution at position 175 is also found in a native sunflower gene variant[11].

[9] *bla* gene - A375, A380, A385, A387, A481
[10] *gus* gene - A378, A387, A436
[11] Accession number XP_022022563.1

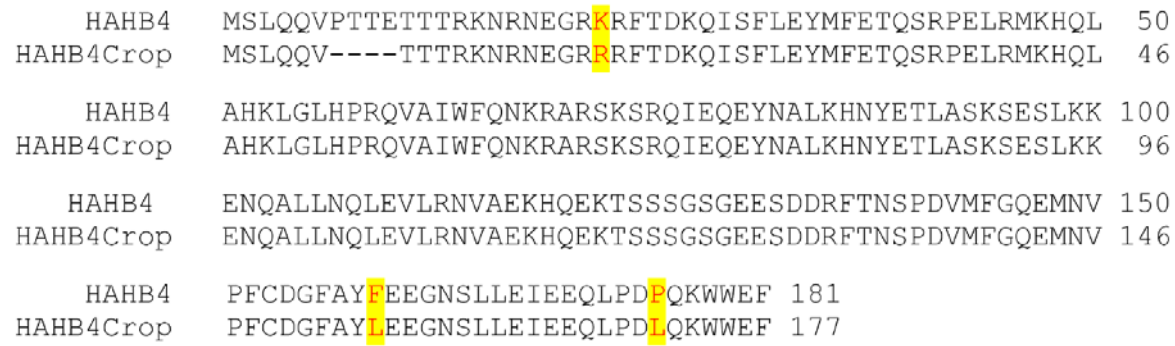


Figure 3: Amino acid sequence alignment of HaHB4 in sunflower and IND-00412-7 (HAHB4Crop). Dashes represent amino acid deletions. Red text and yellow highlight represent amino acid substitutions.

The *HaHB4* gene prepared by the applicant encodes a protein of 177 amino acids, with an expected mass of ~20.9 kDa.

4.1.1 Expression of HaHB4 in IND-00412-7 tissue

As a transcription factor, HaHB4 is most likely expressed at very low levels. Mass spectrometry was used to detect HaHB4 in wheat line IND-00412-7 as standard molecular methodologies lack the required sensitivity (Skinner et al., 2013; Gerber et al., 2003).

The mass spectrometry analysis was performed on seed samples from IND-00412-7 and the non-GM Cadenza control. Samples were obtained from field trials in three different locations in Argentina. Steps were taken to maximise the recovery of proteins and sensitivity of the mass spectrometry detection. *E. coli*-derived HaHB4 (Section 4.1.2) was spiked in the Cadenza control sample for an analytical reference standard. The results showed that grain (seeds) from wheat line IND-00412-7 do not contain detectable or measurable levels of HaHB4[12].

The applicant further examined the expression of HaHB4 in a growth chamber, where conditions of environmental (osmotic) stress can be controlled. Experiments were performed on IND-00412-7 and Cadenza seedlings exposed to either NaCl or mannitol. Protein was extracted from leaf tissue and mass spectrometry was performed. The *E. coli*-derived HaHB4 was used as an analytical reference standard. While HAHB4 could be detected in plants exposed to osmotic stress, the levels were too low for accurate quantification.

These results confirm that the levels of HaHB4 expression in grain and leaf are very low, consistent with the expression of native transcription factors.

4.1.2 Characterisation of HaHB4 expressed in bacteria and its suitability for use in safety assessments

The equivalence of the IND-00412-7 and *E. coli*-derived HaHB4 must be established before the safety data generated using *E. coli*-derived HaHB4 can be applied to IND-00412-7-derived HaHB4. Due to the low levels of transcription factors, a direct comparison could not be made. However, the applicant provided the results of a series of analytical techniques that characterises the *E. coli*-derived HaHB4. The results are summarised below.

[12] The limit of detection and limit of quantification for HaHB4 was 0.01 µg/g and 0.03 µg/g dw seed, respectively.

Molecular weight. Purified *E. coli*-derived HaHB4 was run on SDS-PAGE then visualised with a Colloidal Blue staining kit. The HaHB4 band migrates to ~23 kDa, which is equivalent to the expected mass of IND-00412-7-derived HaHB4.

Matrix-assisted laser desorption/ionization (MALDI-TOF) analysis of HaHB4 samples examined by mass spectrometry was used to determine protein mass. The expected molecular mass was detected.

Immunoreactivity. Western blot analysis with a rabbit polyclonal HaHB4-specific antibody detected a single HaHB4 protein in the *E. coli* preparation with a molecular weight of ~23 kDa.

N-terminal sequencing. Amino acids 1-7 of *E. coli*-derived HaHB4 were sequenced. This analysis confirmed there was no N-terminal modification, no polyhistidine tag (used for protein purification and subsequently removed), and the sequence was identical to the IND-00412-7 derived HaHB4.

Peptide mapping. *E. coli*-derived HaHB4 was digested with trypsin and analysed via liquid chromatography mass spectrometry (LC-MS). Seven peptides were identified and mapped. Sequence coverage was 47% of the expected HaHB4 sequence. The PoteinProphet tool assigned a 99% probability the HaHB4 sequence was correctly identified.

The results outlined in this section demonstrated that *E. coli*-derived HaHB4 is structurally equivalent to IND-00412-7-derived HaHB4. Furthermore, sequence alignment of the translated *E. coli*-derived HaHB4 is identical to the protein sequence of IND-00412-7-derived HaHB4, translated from the inserted DNA sequence. The glycosylation analysis for the HaHB4 sequence would be applicable for both *E. coli*-derived and IND-00412-7-derived HaHB4 (Section 4.1.3). Based on these data, the two proteins are expected to be biochemically and functionally equivalent. It can be concluded that *E. coli*-derived HaHB4 is a suitable surrogate for IND-00412-7-derived HaHB4 for use in the safety assessment experiments described below.

4.1.3 Safety of the introduced HaHB4

Data were provided to assess the potential toxicity and allergenicity of HaHB4.

Bioinformatic analyses of HaHB4

The HaHB4 amino acid sequence was compared to known allergenic proteins in the Allergen Online database and the Structural Database of Allergenic Proteins, as outlined in Section 3.4.3.1[13]. This did not identify any known allergens with significant similarity to HaHB4.

The HaHB4 amino acid sequence was compared with sequences in the Toxin and Toxin Target Database and the Toxin-antitoxin database, as outlined in Section 3.4.3.2. This did not identify any known toxins with significant similarity to HaHB4.

Susceptibility of HaHB4 to digestion

E. coli-produced HaHB4 was incubated with pepsin (10U enzyme/µg protein) for 0-60 min at 37°C. Reactions occurred under acidic conditions in simulated gastric fluid (Thomas et al., 2004). The positive control was the digestible bovine serum albumin (BSA) and the negative control was the non-digestible soybean trypsin inhibitor incubated with pepsin for 0-60 min. A

[13] These databases include allergenic sequences found in commonly consumed food that have homologous sequences to HaHB4, e.g. golden kiwifruit.

no test protein control (pepsin only) and no pepsin control (test protein only) was also used. The extent of digestion was visualised by SDS-PAGE and Coomassie Blue staining.

Visual inspection of the pepsin digestion showed that by 0.5 min, there was no intact HaHB4 remaining in the reaction mix. The BSA control was rapidly digested by 0.5 min, while the soybean trypsin inhibitor remained present over the course of the reaction. These data indicate that HaHB4 will be fully degraded by gastric enzymes in the human digestive system.

Structural stability of HaHB4 after exposure to heat

E. coli-produced HaHB4 was heated for 10, 30 or 60 min at temperatures ranging from 60-90°C. Control samples at 4°C and room temperature were used in the analysis. Control and heated protein samples were run on SDS-PAGE and examined by ELISA to detect the extent of protein degradation, i.e. structural stability. No significant degradation or decrease in signal intensity was observed for HaHB4 in the control, 60, 75 and 90°C treated samples at 10, 30 and 60 min. These data indicate that HaHB4 is not significantly degraded at temperatures up to 90°C.

Although HaHB4 retains structurally stable following heat treatment, this is not directly predictive of allergenicity or toxicity potential. The HaHB4 protein and its homologues are present in food that have a history of prior human consumption. Furthermore, the bioinformatic analysis demonstrated the protein does not have any significant amino acid similarity to known allergens or protein toxins and the digestibility studies suggest that HaHB4 would be rapidly degraded following ingestion.

Post-translational modification

Due to the low expression levels of transcription factors, post-translational modification of HaHB4 could not be directly evaluated. Instead, the IND-00412-7-derived HaHB4 protein sequence was examined *in silico* using algorithms that detect sequences required for glycosylation. These analyses searched for the signal sequence required for protein transport to the endoplasmic reticulum and glycosylation-acceptor sites. No matches were found, indicating that the glycosylation of HaHB4 in wheat is unlikely.

4.1.4 Conclusion

The HaHB4 transcription factor is derived from sunflower and also shares homology with transcription factors found in other commonly consumed foods, indicating a prior history of safe human exposure. Expression studies confirmed very low expression levels of HaHB4 in IND-00412-7 tissue, similar to native transcription factors. A range of characterisation studies were performed on *E. coli*-produced HaHB4 confirming its suitability for use in the safety assessment experiments. While the HaHB4 protein was stable at temperatures of up to 90°C, the protein was susceptible to pepsin digestion and bioinformatic analyses showed HaHB4 had no significant homology to known toxins and allergens. Taken together, this indicates that the HaHB4 protein is unlikely to be toxic or allergenic to humans.

4.2 PAT

The *bar* gene from *S. hygroscopicus* encodes the PAT enzyme and confers tolerance to the antibiotic called bialaphos (Murakami et al., 1986; Thompson et al., 1987). This antibiotic is also produced by *S. hygroscopicus* i.e. the bacterium has evolved a mechanism to avoid the toxicity of its own product (Hara et al., 1991). Bialaphos, now also used as a non-selective herbicide, is a tripeptide comprising two L-alanine residues and an analogue of glutamate known as L-phosphinothricin (L-PPT) (see Thompson et al., 1987) more recently known also

as glufosinate ammonium. Free L-PPT released from bialaphos by peptidases (or applied directly as a synthetic herbicide) inhibits glutamine synthetase which in turn leads to rapid accumulation of ammonia and subsequent cell death.

The PAT protein encoded by the *bar* gene from *S. hygroscopicus* is homologous to the *pat* gene from *S. viridochromogenes*. They both are acetyl transferases with enzyme specificity for both L-PPT and demethylphosphinothricin (DMPT) in the acetylation reaction (Thompson et al., 1987). In the presence of acetyl-Coenzyme A (CoA), PAT catalyses the acetylation of the free amino group of L-PPT to N-acetyl-L-PPT, a herbicidally-inactive compound. The kinetics and substrate specificity of the PAT enzyme are well characterised; it has a high specificity for L-PPT and has been shown to have a very low affinity to related compounds and amino acids; even excess glutamate is unable to block the L-PPT-acetyltransferase reaction (Thompson et al., 1987). The proteins from the two different sources have a sequence identity of 85% (Wehrmann et al., 1996).

The commercialisation of plants engineered for glufosinate-tolerance using PAT began in the mid-1990s (CERA 2011). The history of use of the enzyme in crops therefore extends about 25 years, with FSANZ having assessed and approved 9 events across five commodities with *bar* encoded glufosinate-tolerance and 28 events in total for glufosinate-tolerance.

4.2.1 Expression of PAT in IND-00412-7 tissue

Protein expression in plant tissues was determined by ELISA. An analytical reference standard for plant-derived PAT was generated using a recombinant PAT protein.

In order to determine the sites of accumulation of the protein, samples were collected from IND-00412-7 grown in six field-trial sites in Argentina during the 2013 growing season. Various tissues were examined from IND-00412-7 and the non-GM parental control (Cadenza). Specific tissues were collected at different growth stages. For each tissue sample analysed, four samples were processed from each field-trial site. The field trial sites are as follows: Corral de Bustos (D13); Monte Buey (A13); Villa Saboya (F13); Daireaux (H13); San Jorge (P13); and Balcarce (I13).

The results from the protein analysis showed the maximum levels found in grain were 3.79 µg/g of fresh weight (fw), 11.55 µg/g fw in leaves and 12.67 µg/g fw in stems (Table 6). The level of PAT in root tissue was below the lower limit of quantification. There was no detection of PAT in the control. This result is as expected because the control does not contain the *bar* gene.

Table 6: Expression of PAT (µg/g fw[1]) in various tissues

Tissue	Growth Stage[2]	Site	IND-00412-7	
			Mean	SE[2]
Leaf	Tillering	D13	10.11	1.17
		A13	10.17	0.48
		F13	11.55	0.94
		H13	6.51	0.72
		P13	11.06	1.31
		I13	4.28	0.30
Leaf	Stem elongation	D13	7.11	0.62
		A13	6.82	0.24
		F13	6.61	0.49
		H13	5.82	0.23
		P13	11.36	0.67
		I13	5.36	0.59

Tissue	Growth Stage[2]	Site	IND-00412-7	
			Mean	SE[2]
Root	Heading	D13	0	0
		A13	0	0
		F13	0	0
		H13	0	0
		P13	0	0
		I13	0	0
Stem	Heading	D13	6.74	0.65
		A13	8.72	0.88
		F13	10.80	0.71
		H13	6.59	0.77
		P13	12.67	0.66
		I13	5.36	0.66
Grain	Maturity	D13	3.63	0.50
		A13	3.79	0.35
		F13	3.24	0.32
		H13	2.11	0.32
		P13	3.38	0.40
		I13	1.78	0.13

1. fw - fresh weight. 2. SE – standard error

4.2.2 Characterisation of PAT expressed in IND-00412-7

The *bar* gene prepared by the applicant encodes a protein of 183 amino acids. The protein sequence is perfectly matched to the expected sequence from *S. hygroscopicus* and is the same sequence used in previous applications assessed and approved by FSANZ. It is therefore expected that the PAT protein found in IND-00412-7 is structurally and biochemically similar to PAT found in other plants or from bacteria.

In terms of function, the expression of PAT protein in IND-00412-7 provides the wheat with tolerance to glufosinate. This was initially demonstrated during the transformation and selection process (Section 3.1). During field conditions, the expression of PAT is sufficient to provide IND-00412-7 with tolerance to the glufosinate herbicide (Section 5).

4.2.3 Safety of the introduced PAT

The PAT protein, encoded by either the *pat* or *bar* genes (Hérouet et al., 2005; Wehrmann et al., 1996), has now been considered in 28 FSANZ safety assessments[14]. These assessments, together with the published literature, firmly establish the safety of PAT and confirm that it does not raise toxicity or food allergenicity concerns in humans (ILSI 2016; Hammond et al., 2011; Delaney et al., 2008; Hérouet et al., 2005).

In previous FSANZ assessments, studies on potential allergenicity and toxicity were submitted and assessed. These previous assessments did not raise any safety concerns and there have been no credible reports of adverse health effects in humans. Since the sequence of the protein expressed in IND-00412-7 is identical to the previous PAT sequences assessed by FSANZ, no further safety evaluation is required other than the examination of updated bioinformatic searches.

[14] A372, A375, A380, A385, A386, A446, A481, A518, A533, A543, A589, A1028, A1040, A1046, A1073, A1080, A1081, A1087, A1094, A1106, A1112, A1116, A1118, A1140, A1143, A1192, A1198, A1202.

Bioinformatic analyses of PAT

The applicant has submitted updated bioinformatic studies for PAT that looked for amino acid sequence similarity to known protein allergens and toxins (March 2021). FSANZ has assessed the data submitted by the applicant and the results do not alter conclusions reached in previous assessments.

4.2.4 Conclusion

The data presented by the applicant confirms the PAT expressed in IND-00412-7 is identical to previously assessed PAT proteins. IND-00412-7-derived PAT is immunoreactive to a PAT antibody and is functional, i.e. provides glufosinate tolerance. The protein is expressed in various plant tissues, including grain. Updated bioinformatic analyses confirm that PAT has no similarity with known allergens or toxins that is of significance or concern.

4.3 Herbicide metabolites

FSANZ has assessed the novel herbicide metabolites for glufosinate in GM crops in multiple previous applications. These previous assessments indicate the spraying of IND-00412-7 with glufosinate ammonium would result in the same metabolites that are produced in non-GM wheat sprayed with the same herbicide. As no new glufosinate metabolites would be generated in wheat event IND-00412-7, further assessment is not required.

5 Compositional analysis

The main purpose of compositional analysis is to determine if, as a result of the genetic modification, an unexpected change has occurred to the food. These changes could take the form of alterations in the composition of the plant and its tissues and thus its nutritional adequacy. Compositional analyses can also be important for evaluating the intended effect where there has been a deliberate change to the composition of the food.

The classic approach to the compositional analysis of GM food is a targeted one. Rather than analysing every possible constituent, which would be impractical, the aim is to analyse only those constituents most relevant to the safety of the food or that may have an impact on the whole diet. Important analytes therefore include the key nutrients, toxicants and anti-nutrients for the food in question. The key nutrients and anti-nutrients are those components in a particular food that may have a substantial impact in the overall diet. They may be major constituents (fats, proteins, carbohydrates or enzyme inhibitors such as anti-nutrients) or minor constituents (minerals, vitamins). Key toxicants are those toxicologically significant compounds known to be inherently present in an organism, such as compounds whose toxic potency and level may be significant to health.

5.1 Key components

The key components to be analysed for the comparison of transgenic and conventional wheat are outlined in the OECD Consensus Document on Compositional Considerations for New Varieties of wheat (OECD 2003a). As a minimum, the key nutrients of whole grain appropriate for a comparative study include the proximates, amino acids and vitamins (B vitamins, α-Tocopherol) and the anti-nutrient phytic acid. In addition, fatty acids may be considered.

5.2 Study design

The applicant provided two compositional analysis studies:

1. non-glufosinate treated wheat line IND-00412-7 during the 2012-2013 growing season; and
2. non-glufosinate treated and glufosinate treated wheat line IND-00412-7 during the 2015 growing season.

As the second study included glufosinate treated wheat, it is considered more representative of actual growing conditions for the GM wheat. FSANZ's assessment described below therefore focussed on the 2015 study. FSANZ also examined the 2012-2013 study, the results of which (data not included in this report) are consistent with the 2015 study.

IND-00412-7 was grown and harvested from three field trial sites in Argentina during the 2015 growing season[15]. The sites were representative of environmentally diverse and major wheat production areas. The materials tested in the field trials included IND-00412-7 generation T9 (with or without glufosinate treatment), the non-GM conventional control (Cadenza) and five different reference varieties (Baguette 30, Bio Inta 3006, Sy 100, Baguette 601, Nogal). The field sites were established in a randomised complete block design with four replicates per site.

The analysis of analytes in grain was based on internationally recognised procedures including official methods specified by the Association of Official Analytical Chemists (AOAC), the American Association of Cereal Chemists (AACC), methods specified by the manufacturer of the equipment used for analysis, or other published scientific methods or equations.

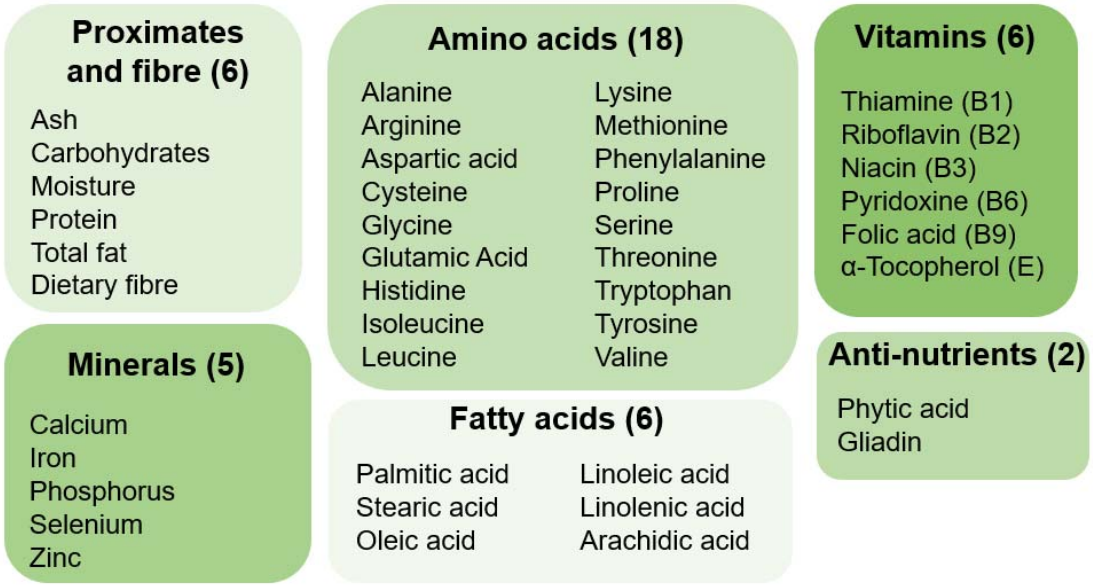
Forty three different analytes were measured in grain (listed in Table 7). For each analyte, the results were expressed as the mean ± the standard error (SE) and range of the four replicates from each location for IND-00412-7 (with or without glufosinate treatment) and the conventional control. Statistically significant differences were analysed using a two-way ANOVA and the least significant difference post-test using the InfoStat software[16].

In assessing the significance of any difference between IND-00412-7 and the control, a P-value of 0.05 was used for all sites (combined-site analysis). Any statistically significant differences were evaluated further on a location basis. Replicates of each reference variety were pooled in each location before analysis, and the results were used to provide the reference range i.e. the natural variability of analytes in a plant grown under the same agronomical and environmental conditions. The natural variation of analytes from publically available data was also considered (OECD 2003a)[17]. This takes into account variability present in non-GM wheat cultivars due to a wide range of agronomic and environment conditions, as well as different genetic backgrounds. These data ranges assist with determining whether any statistically significant differences were likely to be biologically meaningful.

Key analyte levels were also analysed in forage but the results are not included in this report. It is noted however that, in the combined site analysis, the levels of only one analyte (ash) in IND-00412-7 (with or without glufosinate) differed significantly from those of the control. However the mean values of IND-00412-7 (with or without glufosinate) fall within the natural variability seen in the references ranges. This difference is not biologically significant. No other differences were found in the combined-sites analysis.

[15] The location of the three field trial sites: Monte Buey, Córdoba; Pergamino, Buenos Aires; Roldán, Santa Fe.
[16] http://www.infostat.com.ar/
[17] Other datasets exist for analytes in wheat, hence the range of natural variability is expected to be larger.

Table 7: Analytes measured in the grain samples



5.3 Analyses of key components in wheat

5.3.1 Proximates and fibre

A statistically significant difference was observed in IND-00412-7 (glufosinate treated) compared to the control for protein (Table 8). However, this statistically significant difference was not observed in individual field sites. Furthermore, the observed IND-00412-7 mean in the combined site analysis falls well within the variance seen in the reference lines grown under the same conditions and the publically available data. This difference is not biologically significant.

No other statistically significant differences in proximates and fibre were observed between IND-00412-7 and the control (Table 8). Means were also within the natural variability of analytes in the reference varieties and publically available data.

Table 8: Comparison of proximates and fibre

Analyte	Control	IND-00412-7	Herbicide-treated IND-00412-7	Non-GM reference varieties	Publically available data
	Mean (SE) range	Mean (SE) range	Mean (SE) Range	Range	Range
Ash (% dw[1])	2.46 (0.05) 2.17-2.71	2.50 (0.06) 2.29-3.03	2.46 (0.06) 2.11-2.88	1.76-2.32	1.2-3.0
Carbohydrates (% dw)	66.8 (0.3) 65.4-68.2	68.0 (0.3) 66.4-69.5	68.5 (0.3) 66.8-70.0	65.5-69.7	65.4-78.0
Moisture (% fw[2])	12.90 (0.13) 12.20-13.60	12.82 (0.10) 12.40-13.60	12.73 (0.15) 12.07-13.60	12.65-13.9	8.0-18.0
Protein (% dw)	15.8 (0.4) 13.6-19.1	14.9 (0.4) 12.7-16.5	14.1 (0.2) (12.3-15.1)	13.6-17.5	10.0-16.0
Total fat (% dw)	1.5 (0.1) 1.3-2.2	1.5 (0.1) 1.1-1.7	1.5 (0.1) 1.2-1.7	1.0-2.1	1.5-2.0
Dietary fibre (% dw)	13.4 (0.5) 11.1-16.1	13.1 (0.4) 11.7-16.5	13.5 (0.3) 12.1-15.3	11.4-15.6	11.0-14.6

Cells highlighted in blue show statistically significant differences. 1. Dry weight; 2. Fresh weight

5.3.2 Amino acids

A statistically significant difference was observed in IND-00412-7 (without glufosinate treatment) compared to the control for leucine (Table 9). However, this statistically significant difference was not observed in individual field sites. Furthermore, the observed IND-00412-7 mean in the combined site analysis falls well within the variance seen in the reference lines grown under the same conditions and the publically available data. This difference is not biologically significant.

No other statistically significant differences in amino acids were observed between IND-00412-7 and the control (Table 9). Means were also within the natural variability of analytes in the reference varieties and / or publically available data.

Table 9: Comparison of amino acids (% total protein)

Analyte	Control	IND-00412-7	Herbicide-treated IND-00412-7	Non-GM reference varieties	Publically available data
	Mean (SE) range	Mean (SE) range	Mean (SE) Range	Range	Range
Alanine	3.60 (0.13) 2.70-4.40	3.31 (0.13) 2.30-4.20	3.53 (0.13) 3.10-4.70	2.50-3.90	3.4-3.7
Arginine	3.88 (0.12) 2.90-4.40	3.87 (0.19) 2.70-5.30	3.93 (0.15) 2.90-4.70	3.00-4.90	4.0-5.7
Aspartic Acid	4.75 (0.25) 3.70-6.50	5.36 (0.29) 4.20-7.00	5.06 (0.18) 4.10-6.10	4.00-6.50	4.8-5.6
Cysteine	2.54 (0.16) 1.80-3.30	2.60 (0.13) 2.00-3.30	2.77 (0.15) 2.20-3.70	2.10-3.60	1.7-2.7
Glycine	3.48 (0.18) 2.60-4.40	3.19 (0.23) 2.20-4.60	3.33 (0.20) 1.80-4.40	2.70-4.00	3.8-6.1
Glutamic Acid	28.98 (0.68) 25.60-32.80	27.94 (0.34) 26.00-30.20	28.87 (0.55) 25.80-32.10	24.70-32.70	29.9-34.8
Histidine	2.63 (0.16) 1.60-3.60	2.37 (0.10) 1.70-2.90	2.88 (0.12) 2.20-3.40	2.00-3.30	2.0-2.8
Isoleucine	3.11 (0.16) 2.20-3.90	3.33 (0.12) 2.50-4.20	3.48 (0.19) 2.60-4.80	2.30-3.80	3.0-4.3
Leucine	6.07 (0.44) 3.90-8.50	7.11 (0.68) 3.80-9.50	6.78 (0.59) 3.30-10.20	4.70-8.20	5.0-7.3
Lysine	2.85 (0.39) 1.70-5.40	3.14 (0.33) 1.40-4.70	2.77 (0.35) 1.60-4.80	1.40-4.40	2.2-3.0
Methionine	1.59 (0.12) 1.10-2.30	1.83 (0.17) 0.90-2.90	1.87 (0.19) 1.00-2.70	0.90-2.50	1.3-1.7
Phenylalanine	4.28 (0.18) 3.50-5.40	4.16 (0.26) 2.80-5.60	4.31 (0.20) 3.00-5.40	2.70-6.20	3.5-5.4
Proline	7.73 (0.31) 6.50-9.60	8.86 (0.46) 6.60-11.70	8.23 (0.26) 6.60-9.60	6.60-10.60	9.8-11.6
Serine	3.43 (0.22) 2.60-4.70	3.40 (0.13) 2.80-4.30	3.40 (0.18) 2.30-4.60	2.10-5.80	4.3-5.7
Threonine	2.64 (0.22) 1.50-4.00	2.81 (0.24) 1.90-4.10	2.43 (0.18) 1.50-3.50	1.60-3.60	2.4-3.2
Tryptophan	2.02 (0.16) 1.40-3.20	1.77 (0.02) 1.70-1.90	1.85 (0.06) 1.40-2.00	1.40-2.70	1.0-2.1
Tyrosine	2.89 (0.11) 2.30-3.60	2.54 (0.17) 1.60-3.30	2.61 (0.13) 1.90-3.30	1.90-2.90	1.8-3.7
Valine	3.17 (0.10) 2.60-3.60	3.43 (0.20) 2.70-5.00	3.40 (0.10) 3.00-4.20	2.40-5.40	4.4-4.8

Cells highlighted in blue show statistically significant differences.

5.3.3 Vitamins

There were no statistically significant differences found in the level of vitamins in IND-00412-7 compared to the control (Table 10). Means were also within the natural variability of

analytes in the reference varieties and / or publically available data.

Table 10: Comparison of vitamins (mg/kg dw)

Analyte	Control	IND-00412-7	Herbicide-treated IND-00412-7	Non-GM reference varieties	Publically available data
	Mean (SE) range	Mean (SE) range	Mean (SE) Range	Range	Range
Thiamine (B1)	5.2 (0.3) 3.9-7.0	5.2 (0.3) 3.4-6.7	5.3 (0.2) 4.1-6.3	4.0-7.4	1.3-9.9
Riboflavin (B2)	0.43 (0.06) 0.20-0.77	0.56 (0.05) 0.32-0.76	0.47 (0.06) 0.20-0.84	0.24-0.57	0.6-3.1
Niacin (B3)	68.9 (2.8) 53.7-85.2	57.0 (3.1) 39.1-72.3	63.8 (3.8) 45.1-90.4	59.5-73.2	22.0-111.0
Pyridoxine (B6)	6.2 (0.3) 3.8-7.7	4.2 (0.2) 3.3-5.7	5.2 (0.3) 3.7-6.7	3.1-6.7	0.9-7.9
Folic acid (B9)	0.15 (0.01) 0.11-0.19	0.16 (0.01) 0.13-0.19	0.15 (0.01) 0.11-0.19	0.10-0.19	0.2-0.9
α-Tocopherol (E)	13.3 (0.9) 7.1-17.2	12.7 (0.8) 8.8-17.8	14.8 (1.0) 9.2-18.2	7.0-14.4	9-18

Cells highlighted in blue show statistically significant differences.

5.3.4 Minerals

A statistically significant difference was observed in IND-00412-7 (glufosinate treated) compared to the control for zinc (Table 11). However, this statistically significant difference was not observed in individual field sites. Furthermore, the observed IND-00412-7 mean in the combined site analysis falls well within the variance seen in the reference lines grown under the same conditions and the publically available data. This difference is not biologically significant.

No other statistically significant differences in minerals were observed between IND-00412-7 and the control (Table 11). Means were also within the natural variability of analytes in the reference varieties and / or publically available data.

Table 11: Comparison of minerals (mg/kg dw)

Analyte	Control	IND-00412-7	Herbicide-treated IND-00412-7	Non-GM reference varieties	Publically available data
	Mean (SE) range	Mean (SE) range	Mean (SE) Range	Range	Range
Calcium	483 (22) 396-621	453 (8) 403-501	457 (12) 363-503	350-574	250-538
Iron	47 (2) 38-62	46 (2) 38-62	45 (2) 38-62	31-44	33-79
Phosphorus	4438 (115) 3860-5068	4620 (81) 4259-5247	4573 (141) 3468-5104	3368-5387	3320-5160
Selenium	0.30 (0.03) 0.15-0.49	0.30 (0.02) 0.15-0.45	0.30 (0.02) 0.19-0.41	0.12-0.41	0.04-0.71
Zinc	50 (3) 37-64	42 (3) 26-63	39 (3) 24-55	18-63	24-47

Cells highlighted in blue show statistically significant differences.

5.3.4 Fatty acids

There were no statistically significant differences found in the level of fatty acids in IND-00412-7 compared to the control (Table 12). Means were also within the natural variability of analytes in the reference varieties and / or publically available data.

Table 12: Comparison of fatty acids (% total fatty acid)

Analyte	Control	IND-00412-7	Herbicide-treated IND-00412-7	Non-GM reference varieties	Publically available data
	Mean (SE) range	Mean (SE) range	Mean (SE) Range	Range	Range
Palmitic acid	15.6 (0.2) 13.8-16.7	15.9 (0.2) 14.9-17.5	16.3 (0.2) 15.3-17.8	14.8-19.9	11-32
Stearic acid	1.3 (0.2) 1.0-2.8	1.5 (0.2) 1.0-2.8	1.4 (0.2) 1.0-3.9	0.9-2.0	0-4.6
Oleic acid	20.9 (0.1) 20.3-22.0	20.4 (0.2) 19.5-22.8	20.3 (0.3) 19.4-23.2	16.1-19.5	11-29
Linoleic acid	57.6 (0.4) 55.0-59.2	57.2 (0.5) 53.1-58.8	57.3 (0.7) 51.3-59.3	55.5-59.7	44-74
Linolenic acid	3.6 (0.1) 3.4-4.5	3.9 (0.1) 3.5-4.9	3.7 (0.1) 3.2-4.3	3.6-5.4	0.7-4.4
Arachidic acid	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	–

Cells highlighted in blue show statistically significant differences.

5.3.6 Anti-nutrients

There were no statistically significant differences found in the level of anti-nutrients in IND-00412-7 compared to the control (Table 13). Means were also within the natural variability of analytes in the reference varieties and / or publically available data.

Table 13: Comparison of anti-nutrients (% dw)

Analyte	Control	IND-00412-7	Herbicide-treated IND-00412-7	Non-GM reference varieties	Publically available data
	Mean (SE) range	Mean (SE) range	Mean (SE) Range	Range	Range
Phytic acid	1.5 (0.1) 0.9-2.2	1.4 (0.1) 0.7-2.0	1.4 (0.1) 0.8-2.2	1.1-2.5	0.49-0.93[1]
Gliadin	6.7 (0.1) 6.1-7.5	6.4 (0.2) 5.6-7.1	6.4 (0.1) 5.5-6.9	6.1-7.1	3.9-9.1[2]

Cells highlighted in blue show statistically significant differences. 1. Obert, 2004; 2. Huebner and Rothfus, 1968.

5.4 Conclusion

Mean values were provided for 43 analytes measured in wheat grain. Statistically significant differences were reported for 3 analytes (Table 14). These differences fall within the reference ranges of the commercial non-GM varieties and / or publically available data as described in OECD 2003a. Like any food crop, nutrient and anti-nutrient composition of wheat grain can be impacted by cultivation site, agricultural practices and the environment. The differences reported here are consistent with the normal biological variability that exists in wheat.

Overall, the compositional data are consistent with the conclusion that there are no biologically significant differences in the levels of key constituents in IND-00412-7 when compared with conventional wheat cultivars already available in agricultural markets.

Table 14: Summary of statistically significant compositional differences between control and IND-00412-7

Analyte	Control Mean (range)	IND-00412-7 Mean (range)	Are values within the reference and / or publically available data ranges? Yes / No
Protein (% dw)	15.8 13.6-19.1	14.1[1] 12.3-15.1	Yes
Leucine (% total protein)	6.07 3.90-8.50	7.11[2] 3.80-9.50	Yes
Zinc (mg/kg dw)	50 37-64	39[1] 37-64	Yes

Cells highlighted in purple show data where IND-00412-7 is significantly lower than the control and cell highlighted in green show data where IND-00412-7 is significantly higher than the control. 1. Treated with glufosinate; 2. Without glufosinate treatment.

6 Nutritional impact

In assessing the safety of a GM food, a key factor is the need to establish that the food is nutritionally adequate and will support typical growth and wellbeing. In most cases, this can be achieved through a detailed understanding of the genetic modification and its consequences, together with an extensive compositional analysis of the food, such as that presented in Section 5 of this report.

Where a GM food has been shown to be compositionally equivalent to conventional varieties, the evidence to date indicates that feeding studies using target livestock or other animal species will add little to the safety assessment (Bartholomaeus et al., 2013; OECD 2003b). If the compositional analysis indicates biologically significant changes, either intended or unintended, to the levels of certain nutrients in the GM food, additional nutritional studies should be undertaken to assess the potential impact of the changes on the whole diet.

IND-00412-7 is the result of genetic modifications to confer tolerance to drought and the herbicide glufosinate, with no intention to significantly alter nutritional parameters in the food. The compositional analyses have demonstrated that the genetic modifications have not altered the nutrient composition of IND-00412-7 compared with that of conventional non-GM wheat varieties. The introduction of food derived from IND-00412-7 into the food supply is therefore expected to have negligible nutritional impact.

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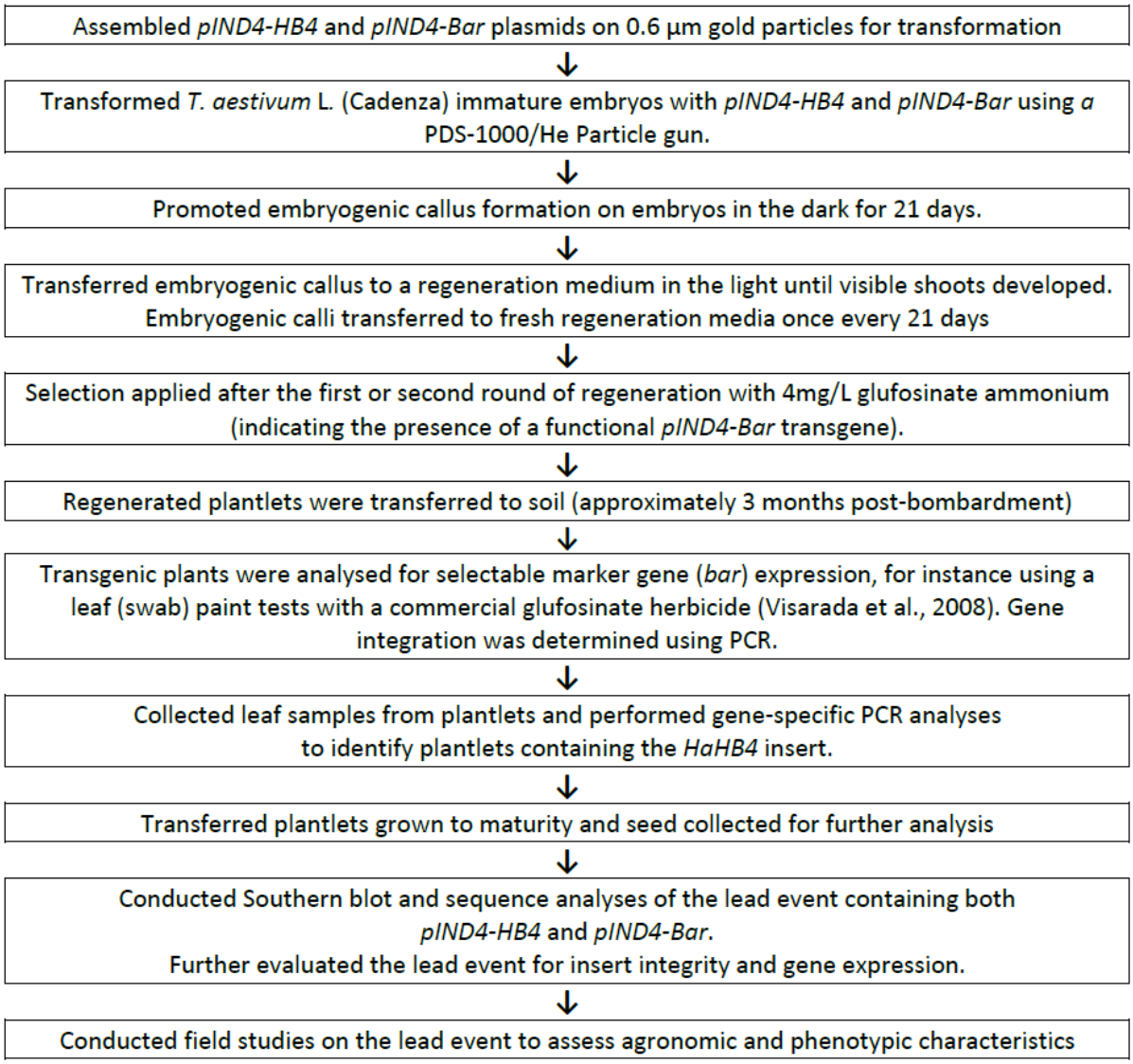
Appendix 1

Flowchart showing the development process in the creation of the IND-00412-7 wheat line



Appendix 2

Representation of the development of IND-00412-7 and the generations used in the characterisation studies