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416-26

Supporting document

Safety assessment - Application A1356

Food derived from increased nicotianamine wheat lines
containing event UM-12115T-1

Executive summary

Application A1356 seeks approval for the sale and use of food derived from wheat lines containing event UM-12115T-1, genetically modified (GM) to have increased levels of nicotianamine (NA).

NA occurs naturally in plants where it is involved in the uptake and transport of transition metals such as zinc and iron. The levels of NA were increased by inserting the *OsNAS2* gene from rice (*Oryza sativa*), which encodes the OsNAS2 nicotianamine synthase protein. The genetic modification also introduced the *hph* gene from *Escherichia coli* encoding the hygromycin phosphotransferase (HPT) protein, which was used as a selectable marker.

Food Standards Australia New Zealand (FSANZ) has previously assessed the HPT protein. This is the first time FSANZ has assessed the OsNAS2 protein.

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 has a long history of safe use as food for most people except those who are wheat allergic or have coeliac disease. Wheat-derived products are routinely used in a large number and diverse range of foods e.g. flour, baked goods and breakfast cereals.

Molecular characterisation

The genes encoding OsNAS2 (*OsNAS2*) and HPT (*hph*) were introduced into wheat via *Agrobacterium*-mediated transformation. Detailed molecular analyses indicated two independent insertions into the wheat genome. Both insertion loci contain a single copy of inserted DNA containing the *OsNAS2* and *hph* expression cassettes.

One locus also contains a fragment of the plasmid backbone containing the *nptIII* kanamycin resistance gene, a bacterial origin of replication (*ori*) and basis of mobility (*bom*) sequence. Due to the lack of eukaryotic regulatory elements, the *nptIII* gene is not expressed in wheat. In addition, because wheat lacks the bacterial molecular machinery required for plasmid replication and transfer, the *ori* and *bom* sequences are non-functional.

The introduced genetic elements were shown by molecular techniques and phenotypic analyses to be stably inherited across multiple generations.

Characterisation and safety assessment of new substances

The OsNAS2 protein was expressed in both the leaf tissue (0.32 ng/mg) and grain (flour) (0.024 ng/mg) of UM-12115T-1 wheat. The OsNAS2 protein is derived from rice, and along with other similar proteins found in cereal crops, has a long history of human consumption. OsNAS2 is rapidly digested by pepsin and is heat labile, indicating it would be thoroughly degraded following ingestion. Bioinformatic analyses showed OsNAS2 does not share any meaningful homology with known allergens, coeliac disease-associated proteins, or toxins.

The HPT protein was below the limit of detection in leaf and flour, indicating that exposure to HPT from consumption of UM-12115T-1 wheat would be negligible. Characterisation studies confirmed the HPT protein is identical to HPT previously assessed by FSANZ. Updated bioinformatic analyses for HPT were consistent with previous analyses showing this protein has no significant homology with any known allergens or toxins.

Taken together, the evidence supports the conclusion that OsNAS2 and HPT are not toxic or allergenic to humans.

Compositional analyses

Detailed compositional analyses of UM-12115T-1 wheat lines were completed. Statistically significant differences were found between grain from UM-12115T-1 wheat and the non-GM control for 3 of the 68 analytes evaluated, however these differences were small and within the range of normal biological variability that exists in wheat.

In relation to NA, levels were significantly higher in UM-12115T-1 grain compared to the non-GM control. However, NA concentrations remained within the range observed among conventional wheat varieties. No significant differences in grain iron or zinc levels were observed between UM-12115T-1 and the non-GM control.

Overall, the compositional data indicates there are no biologically significant differences in the levels of key constituents in grain from UM-12115T-1 wheat compared to grain from non-GM wheat varieties.

Conclusion

No potential public health and safety concerns have been identified in the assessment of increased nicotianamine wheat lines containing event UM-12115T-1. Based on data provided in the present application and other available information, food derived from these wheat lines is as safe for human consumption as food derived from conventional non-GM wheat varieties.

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List of Abbreviations

Abbreviation	Description
aa	amino acid(s)
Bom	basis of mobility
bp	base pair(s)
BSA	bovine serum albumin
CCI	confidential commercial information
DNA	deoxyribonucleic acid
Fe	iron
FSANZ	Food Standards Australia New Zealand
Gbp	Gigabase pairs
GM	genetically modified
HPT	hygromycin phosphotransferase
kDa	kilodalton(s)
LB	left border (sequence)
LC-MS/MS	liquid chromatography tandem mass spectrometry
Mbp	Megabase pairs
mg	milligram(s)
MT	million tonnes
NA	nicotianamine
NAS	nicotianamine synthase
ng	nanogram(s)
OECD	Organisation for Economic Co-operation and Development
OGTR	Office of the Gene Technology Regulator
PCR	polymerase chain reaction
qRT-PCR	quantitative real-time polymerase chain reaction
RB	right border (sequence)
RNA	ribonucleic acid
RF	reading frame
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM	standard error of the mean
SGF	simulated gastric fluid
T-DNA	transfer DNA
WT	wild-type
Zn	zinc

1 Introduction

Food Standards Australia New Zealand (FSANZ) received an application from the University of Melbourne to vary Schedule 26 in the Australia New Zealand Food Standards Code. The variation is to include food from new genetically modified (GM) wheat lines containing event UM-12115T-1, with the OECD Unique Identifier UM-12115T-1. This event results in increased levels of nicotianamine (NA), a non-protein amino acid found naturally in plants and involved in the uptake and transport of transition metals such as iron and zinc.

Increased NA levels are produced by expressing the *OsNAS2* gene from rice (*Oryza sativa*), which encodes the *OsNAS2* nicotianamine synthase protein. UM-12115T-1 also contains the hygromycin phosphotransferase (HPT) protein from *Escherichia coli*, which was used as a selectable marker.

FSANZ has previously assessed the HPT protein. This is the first time FSANZ has assessed the *OsNAS2* protein.

The applicant is currently seeking a licence for the commercial cultivation of wheat lines containing event UM-12115T-1 from the Gene Technology Regulator (GTR¹).

2 History of use

2.1 Host organism

The host organism is bread wheat (*Triticum aestivum* L.). The conventional wheat variety Gladius was used as the parental variety for the genetic modification described in this application.

Bread wheat is one of the world's most important food crops and has been cultivated for more than 6000 years (Shiferaw et al. 2013; Balfourier et al. 2019). In addition to its use as a staple food crop, wheat is widely used as a component of animal feed. Due to its agricultural and economic importance, wheat has been extensively studied, and its biology is well characterised.²

Global wheat production was approximately 799 million tonnes (MT) in 2024 (FAOSTAT 2026). China, India and the Russian Federation were the largest producers, accounting for 140, 113 and 83 MT, respectively. Australia produced approximately 34 MT of wheat in 2024. Wheat production in New Zealand is comparatively limited, with approximately 0.46 MT produced in 2024 (FAOSTAT 2026).

Wheat has a long history of safe use as food for human consumption (Shewry and Hey 2015; OECD 2024). However, wheat can cause adverse reactions in susceptible individuals, including those with wheat allergy or coeliac disease, each of which affects approximately 1% of the population (Liu et al. 2023; Singh et al. 2018). Typically, wheat grain is milled into flour and used in a wide range of food products, including bread, pasta, biscuits and other baked goods.

¹ The Office of the Gene Technology Regulator (OGTR) provides administrative support to the Gene Technology Regulator in the performance of functions under the *Gene Technology Act 2000*.

² Refer to detailed reports published by the OECD (OECD 2024) and the Office of the Gene Technology Regulator (OGTR 2021).

2.2 Donor organisms

2.2.1 *Oryza sativa*

The *OsNAS2* gene is derived from rice (*Oryza sativa*). Like wheat, rice is a staple food crop and has been safely consumed by humans for thousands of years. While food-induced allergic reactions to rice have been reported, these are rare and rice is not considered to be a common allergenic food in Australia and New Zealand³ (Gilissen et al. 2014).

2.2.2 *Escherichia coli*

The *hph* gene is derived from *Escherichia coli*, a Gram-negative bacterium which is ubiquitous in the environment and commonly found in the gastrointestinal tract of humans and animals. Most *E. coli* strains are harmless to humans, although some strains can cause severe illness. *E. coli* is widely used in the commercial manufacturing of products ranging from amino acids and vitamins for food applications, to recombinant human proteins used in pharmaceutical applications, including injectable protein products such as insulin (İncir and Kaplan 2024).

2.2.3 Other organisms

Genetic elements from several other organisms have been used to produce event UM-12115T-1 (refer to Tables 1 and 2 and Appendix 1). These genetic elements are either:

- non-coding sequences that are used to regulate the expression of the inserted *OsNAS2* and *hph* genes
- coding and non-coding sequences from the transformation plasmid.

The potential for the latter sequences to result in unintended protein expression, replication and mobility is addressed in section 3.4.2.

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

To develop wheat event UM-12115T-1, the conventional bread wheat variety 'Gladius' was transformed with a plasmid containing the *OsNAS2* and *hph* expression cassettes within a transfer DNA (T-DNA) region. Immature isolated 'Gladius' embryos were transformed with a strain of *Agrobacterium tumefaciens* containing the plasmid pUbi-*OsNAS2* (Figure 1) using a

³ FSANZ: Food allergies – <https://www.foodstandards.gov.au/consumer/foodallergies>

procedure adapted from Sparks et al. 2014 and Hensel et al. 2017.

Briefly, the embryos were inoculated with an *Agrobacterium* suspension then transferred onto a co-cultivation medium and incubated for 3 days to allow integration of the T-DNA. Transformed embryos were transferred to an induction medium containing an antibiotic to kill *Agrobacterium* and hygromycin B to allow selection of plants containing the *hph* selectable marker gene. After a further 3 to 4 weeks, the resulting calli⁴ were regenerated in the light to stimulate shoot and root development. Plants were then transferred to soil and grown to maturity before further molecular and phenotypic characterisation.

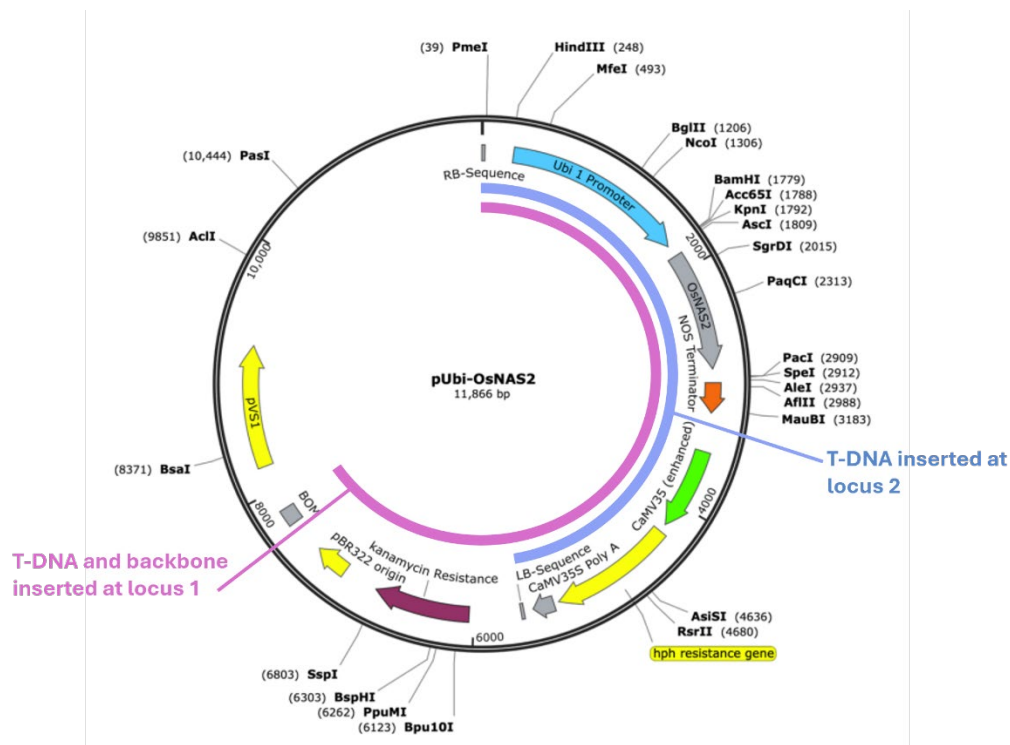


Figure 1. Map of plasmid pUbi-OsNAS2. The T-DNA region containing the *OsNAS2* and *hph* expression cassettes, as well as part of the plasmid backbone containing the kanamycin resistance (*nptIII*) gene, the pBR322 origin of replication and the basis of mobility (*bom*) sequence was inserted into the wheat genome at one locus (purple line). A second insertion locus contained only the T-DNA region between the left border (LB) and right border (RB) sequences (blue line).

3.2 Detailed description of inserted DNA

Information on *OsNAS2* and *hph* genetic elements from pUbi-OsNAS2 introduced during transformation is summarised in Table 1.

⁴ A callus (plural 'calli') is a mass of plant cells in culture, capable of differentiation into a new plant.

Table 1. *OsNAS2* and *hph* expression cassettes from *pUbi-OsNAS2* integrated into UM-12115T-1

	Promoter	Coding sequence	Terminator	Expected Expression Pattern	Notes
Nicotinamine synthase 2 (<i>OsNAS2</i>)	<i>Ubi1</i> promoter (<i>Zea mays</i>)	Nicotinamine synthase 2 (<i>Oryza sativa</i>)	Nopaline synthase (<i>nos</i>) terminator (<i>A. tumefaciens</i>)	Constitutive	Enzyme involved in nicotianamine biosynthesis
Hygromycin phosphotransferase (<i>hph</i>)	Enhanced CaMV35S promoter (Cauliflower mosaic virus)	Hygromycin phosphotransferase (<i>Klebsiella</i> sp. via <i>Escherichia coli</i>)	<i>CMV terminator</i> region (Cauliflower mosaic virus)	Constitutive	Selectable marker (hygromycin resistance)

Backbone and other sequences

Information on the backbone elements from *pUbi-OsNAS2* introduced during transformation is summarised in Table 2.

Table 2. *Backbone elements from pUbi-OsNAS2 integrated into UM-12115T-1*

Genetic element	Source	Description and function
<i>nptIII</i> (encoding 3'5"-aminoglycoside phosphotransferase type III)	<i>Enterococcus faecalis</i> pJH1	Confers kanamycin resistance in microbial hosts during cloning; no function in plants
pBR322 <i>ori</i>	pMDC32 plasmid-derived	Plasmid origin of replication; no function in plants
bom	pMDC32 plasmid-derived	pBR322 basis of mobility sequence; no function in plants

Additional detail, including information on the plasmid backbone elements and intervening sequences used to assist with cloning, mapping and sequence analysis, can be found in [Appendix 1](#).

3.3 Development of the wheat event from the original transformant

A breeding programme was undertaken for the purposes of:

- obtaining generations suitable for analysing the characteristics of wheat lines containing event UM-12115T-1
- ensuring that the UM-12115T-1 event is incorporated into elite lines for commercialisation.

T0 ⊗ → T1 ⊗ → T2 ⊗ → T3 ⊗ → T4 ⊗ → T5 ⊗ → T6 ⊗ → T7 ⊗ → T8

⊗ denotes self-pollination

Figure 2. *Breeding path used in the characterisation of event UM-12115T-1.*

The original transformant (T0 generation) was self-pollinated for multiple generations to produce generations T1-T8 (Figure 2). The generations analysed for the molecular characterisation and other analyses are listed in Table 3.

Table 3. *UM-12115T-1 generations used for various analyses*

Analysis	Section	Generation(s) used
Insertion copy number, insertion organisation, insertion integrity, site of insertion and genetic stability by PacBio HiFi sequencing	Section 3.4.1 ; Section 3.4.2 ; Section 3.4.3	T8
Genetic stability (PCR screening)	Section 3.4.3.1	T1-T4
Phenotypic stability/trait expression	Section 3.4.4	T4-T8
Protein expression; compositional analysis	Section 4 ; Section 5	T8

Event UM-12115T-1 will be crossed into elite wheat varieties through conventional breeding, resulting in commercial wheat varieties for food use. Because Locus 1 (Chromosome 1D) and Locus 2 (Chromosome 4D) are unlinked, they will segregate independently during cross-pollination. As a result, commercial derivatives of UM-12115T-1 may contain both loci, Locus 1 only, or Locus 2 only.

3.4 Characterisation of the inserted DNA and sites of insertion

A range of analyses were undertaken to characterise the genetic modification in UM-12115T-1 wheat lines. These analyses focused on the nature and stability of the insertion and whether any unintended DNA changes may have occurred because of the transformation procedure.

Genomic DNA from the T8 generation of UM-12115T-1 was sequenced using PacBio HiFi long-read sequencing. The sequence data were assembled *de novo* to produce a reference genome sequence. The assembled genome was 14.64 Gigabase pairs (Gbp) in size, which is consistent with the total size of the hexaploid wheat genome (~17 Gbp). This approach enabled complete resolution of the inserted DNA and flanking genomic regions.

3.4.1 Number of integration sites, insert integrity and sites of integration

Alignment of the UM-12115T-1 sequence assembly against the plasmid reference sequence identified 2 distinct insertion loci in the UM-12115T-1 genome, referred to as Locus 1 and Locus 2 in this document. Each site contained a single copy of the inserted DNA.

Locus 1, located on Chromosome 1D⁵, contains a 7,989 base pair (bp) insertion comprising the intended T-DNA as well as an adjacent fragment of plasmid backbone containing the *nptIII* gene, the pBR322 origin of replication (*ori*) and the basis of mobility (*bom*) sequence (Figure 3).

The *OsNAS2* and *hph* expression cassettes at Locus 1 are largely intact. A small truncation is present within the CaMV35S polyadenylation sequence of the *hph* cassette. However, because *hph* functioned successfully as a selectable marker during transformation (section 3.1), the truncation did not prevent expression of the *hph* gene at levels required for selection.

⁵ The bread wheat genome is hexaploid – it contains 6 sets of chromosomes, organised into 3 subgenomes (AABBDD).

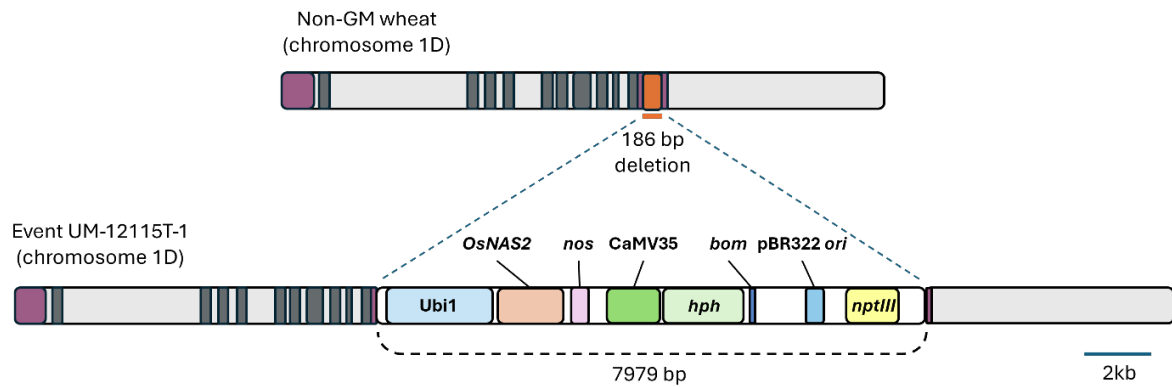


Figure 3. Schematic of the inserted DNA at Locus 1 in event UM-12115T-1. The 7,979 bp insert contains the *OsNAS2* and *hph* expression cassettes along with the *nptIII*, *pBR322 ori* and *bom* plasmid backbone elements. The purple regions represent the UTR of the endogenous *PEAMT1* gene. The dark grey elements represent *PEAMT1* exons.

The adjacent fragment of plasmid backbone was inserted in reverse orientation, with the sequential order of the *bom*, *ori* and *nptIII* elements inverted relative to their original configuration on the transformation plasmid (Figure 3). Rearrangements of this kind are commonly observed during *Agrobacterium*-mediated transformation (Kononov et al. 1997; Salomon and Puchta 1998).

The insertion at Locus 1 occurred within the 3' untranslated region of the endogenous wheat phosphoethanolamine N-methyltransferase 1 (*PEAMT1*) gene. Wheat contains multiple homologous copies of the *PEAMT* gene as well as multiple isoforms of the phosphoethanolamine N-methyltransferase enzyme (Jost et al. 2009). Therefore, the insertion at this site is unlikely to have any functional effect on the plant.

The insertion is accompanied by a 186 bp deletion of the native wheat genome at the insertion site (Figure 3), as is typical in *Agrobacterium*-mediated transformation (Kleinboelting et al. 2015).

Locus 2, located on Chromosome 4D, contains a 5,504 bp insertion consisting of the intended T-DNA sequence. This insert contains the *OsNAS2* and *hph* expression cassettes in the expected configuration, with no plasmid backbone (Figure 4).

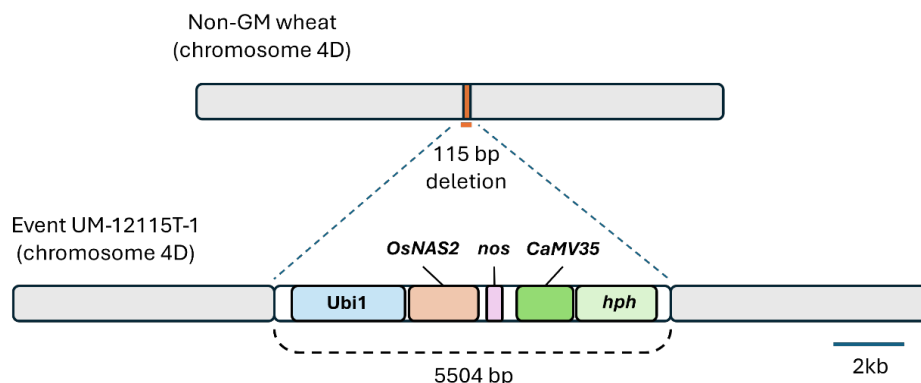


Figure 4. Schematic of the inserted DNA at Locus 2 in event UM-12115T-1. The 5,504 bp insert contains the *OsNAS2* and *hph* expression cassettes.

The insertion is contained within an intergenic region of Chromosome 4D, accompanied by a

115 bp deletion native intergenic DNA (Figure 4). Analysis of the flanking sequences confirmed the junctions between the inserted DNA wheat genomic DNA were intact.

No plasmid-derived sequences were detected in the non-GM control. Sequence alignment to the *OsNAS2* coding region reflected homology with endogenous wheat nicotianamine synthase genes (Bonneau et al. 2016).

3.4.2 Integration of backbone and other sequences

As noted in section 3.4.1, the insertion into the UM-12115T-1 genome at Locus 1 contains the *bom*, *ori* and *nptIII* sequences from the pUbi-OsNAS2 plasmid backbone. Other than these sequences, no additional backbone sequences were identified elsewhere in the genome.

The *nptIII* gene (also known as *aph(3')-IIIa*), originally isolated from an R-plasmid from *Enterococcus faecalis* (Trieu-Cuot et al. 1988; Shaw et al. 1993), encodes ATP:kanamycin 3'-O-phosphotransferase, a bacterial aminoglycoside-modifying enzyme that confers resistance to a broad spectrum of aminoglycoside antibiotics, including kanamycin (Woegerbauer 2007).

Unlike the *nptII* gene, which has a long history of use as a selectable marker in GM plants (Miki and McHugh 2004; EFSA 2004; EFSA 2009), *nptIII* is typically used for plasmid maintenance in bacterial hosts. In event UM-12115T-1, *nptIII* was integrated into the wheat genome from the transformation plasmid backbone (section 3.2). However, because it was located outside the intended expression cassettes, it is not associated with plant promoter or terminator sequences capable of driving expression in plant tissues.

To confirm the absence of expression, flour and leaf tissue from UM-12115T-1 was analysed by mass spectrometry, as described in section 4.1.1. NPTIII was not detected in either the flour or leaf samples. These results indicate the *nptIII* gene is not expressed in UM-12115T-1 and does not require further consideration as a novel protein.

In addition to the *nptIII* gene, the insertion at Locus 1 contains the pBR322 origin of replication (*ori*) and basis of mobility (*bom*) sequences from the plasmid backbone. These sequences are non-coding recognition elements involved in plasmid replication and conjugative DNA transfer in bacteria, respectively, and their activity requires bacterial proteins that are absent from plant cells (del Solar et al. 1998; Finnegan and Sherratt 1979). As these elements are non-coding and non-functional in plants, their presence does not pose a safety concern.

3.4.3 Stability of the genetic changes in wheat lines containing event UM-12115T-1

Stability with respect to the genetic changes refers to both genetic and phenotypic stability of the introduced trait. Genetic stability refers to maintenance of the genetic modification (as produced in the initial transformation events) over successive generations. Phenotypic stability refers to the expressed trait remaining unchanged over successive generations.

3.4.3.1 Genetic stability

A combination of targeted polymerase chain reaction (PCR) screening and PacBio HiFi sequencing was used to demonstrate the genetic stability of event UM-12115T-1 across the T1 to T8 generations.

PCR results demonstrated the introduced genetic material was consistently inherited across

generations, and both insertion loci were retained following fixation of the event. Long-read sequencing of the T8 generation confirmed the integrity of the inserted sequences. Together, these results demonstrate that the genetic modification is stably maintained in UM-12115T-1.

3.4.3.2 Phenotypic stability

OsNAS2 protein was detected in grain and leaf tissues of the T8 generation (see section 4.1.1). Together with the genetic stability data, this confirms that event UM-12115T-1 exhibits stable inheritance and expression of the introduced OsNAS2 gene.

3.4.4 Open reading frame analysis

A bioinformatic analysis of both insertions in event UM-12115T-1, as well as flanking DNA regions, was done to identify whether any novel open reading frames (ORFs) had been created, and if so, whether these sequences have the potential for allergenicity or toxicity. These analyses are theoretical only, as it is highly unlikely that any of the identified ORFs would be expressed *in planta*.

DNA spanning each insertion and the flanking genomic regions was translated in all 6 reading frames from the first ATG (start) codon to the next in-frame stop codon, resulting in 94 unique ORFs (53 for Locus 1 and 41 for Locus 2) of ≥ 30 amino acids (aa) being identified for further analysis.

Homology searches for all 94 ORFs were conducted on the AllergenOnline database⁶ (version 24) using a full-length allergen homology assessment, 80-aa sliding window analysis, and 8-aa exact match search. No RFs showed biologically significant similarity to known allergens. Limited sequence matches were identified for a small number of ORFs in the 80-aa sliding window and 8-aa exact match searches; however, these were restricted to short regions and were not supported by full-length sequence homology.

A machine learning-based allergenicity prediction using NetAllergen-1.0 was also applied to all RFs (Li et al. 2023). Physicochemical flags were observed for 34 ORFs, including the *nptIII* protein, which is known not to be allergenic. Homology screening within the prediction did not identify any RFs as allergens and the physicochemical flags were therefore considered computational false positives.

All 94 ORFs were translated *in silico* and queried against a curated toxin database built by annotating UniProtKB entries with the keyword 'Toxin'. The search used BLASTP with a BLOSUM62 substitution matrix, E-value threshold of 1×10^{-5} and low-complexity filtering. No statistically significant sequence similarities were identified between any RF and any toxin sequence in the curated database and no alignments indicative of toxin-like motifs were observed.

3.5 Conclusion

Event UM-12115T-1 contains two independent insertion loci on chromosomes 1D (Locus 1) and 4D (Locus 2) of the wheat genome. Each locus contains a single copy of the OsNAS2 and *hph* expression cassettes in the expected arrangement, except for a truncation within the CaMV35S polyadenylation sequence of the *hph* cassette at Locus 1. In addition, Locus 1 contains a rearranged fragment of plasmid backbone comprising the *nptIII*, *ori* and *bom* sequences which are non-functional. No additional backbone sequences were detected elsewhere in the genome. The inserted DNA was stably inherited across multiple

⁶ <https://www.allergenonline.org/>

generations, and bioinformatic analyses of novel ORFs generated by the insertions did not raise any allergenicity or toxicity concerns.

4 Characterisation and safety assessment of newly expressed proteins

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, either because of anti-nutrient or allergenic effects (Delaney et al. 2008).

To identify potential hazards, the assessment considered biochemical function, phenotypic effects, and concentration levels of OsNAS2 and HPT in the edible part of the plant, as well as any post-translational modifications that may have occurred.

4.1 OsNAS2

OsNAS2 is a protein of 326 amino acids with a molecular weight of ~34.5 kDa and catalyses the conversion of S-adenosyl-L-methionine (SAM) to NA (Figure 5).

NA is a naturally occurring non-proteinogenic amino acid that is ubiquitous in higher plants (Noma and Noguchi 1976; Takahashi et al. 2003) where it is involved in the transport and distribution of iron (Fe), zinc (Zn) and other divalent metal cations (von Wiren et al. 1999; Takahashi et al. 2003).

In cereals such as wheat, NA is a precursor for the synthesis of mugineic acid family phytosiderophores, which are secreted from roots and facilitate the uptake of ferric iron (Fe^{3+}) from the rhizosphere (Higuchi et al. 1999; Schuler and Bauer 2011).

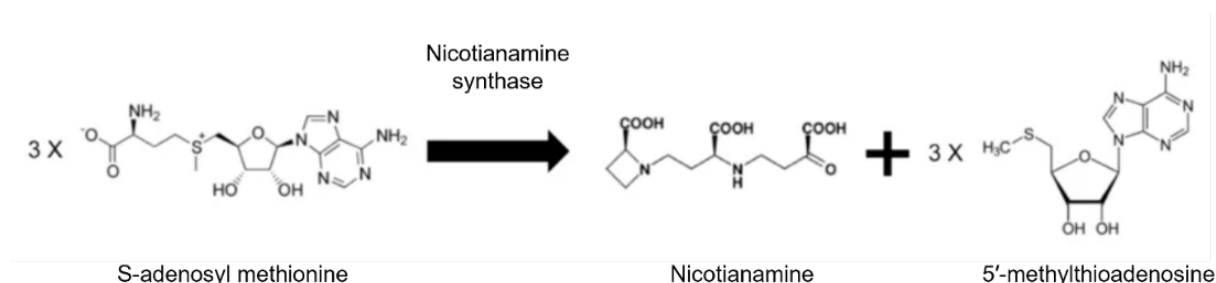


Figure 5. The reaction catalysed by nicotianamine synthase. Three molecules of S-adenosyl-L-methionine are converted into nicotianamine along with three molecules of 5'-methylthioadenosine as a co-product.

Genes encoding NA synthase are widely distributed throughout the plant kingdom, including in rice, barley and maize and share conserved catalytic motifs characteristic of other NA synthase enzymes. Humans therefore have a long history of dietary exposure to nicotianamine synthase through consumption of plant foods.

The OsNAS2 protein was expressed in event UM-12115T-1 with the intent to increase flux through the endogenous NA biosynthesis pathway. Expression of the OsNAS2 gene in rice and other cereals has been shown to increase concentrations of NA and phytosiderophores, which in turn enhance the uptake, translocation and accumulation of Fe and Zn in developing grain (Curie et al. 2009; Lee et al. 2009; Johnson et al. 2011; Schuler and Bauer 2011).

4.1.1 Expression levels of OsNAS2 in event UM-12115T-1

Expression of OsNAS2 in UM-12115T-1 was measured by analysis of both the RNA transcript and its expressed protein.

Quantitative real-time PCR (qRT-PCR) confirmed that the OsNAS2 gene is actively transcribed in UM-12115T-1. As expected, OsNAS2 transcripts were not detected in the non-GM control.

Expression of the introduced OsNAS2 protein was measured in grain (flour) and leaf tissue of UM-12115T-1 using liquid chromatography-tandem mass spectrometry (LC-MS/MS). The mean OsNAS2 concentration was 0.32 ng/mg extractable protein in leaf and 0.024 ng/mg in flour, indicating an approximately 13-fold higher expression of OsNAS2 in vegetative leaf tissue compared to grain. OsNAS2 was not detected in tissue samples from the non-GM control.

4.1.2 Characterisation of microbially expressed OsNAS2

To produce enough purified OsNAS2 for digestibility and heat stability studies, the applicant expressed OsNAS2 in *Escherichia coli*. This was necessary because of the low expression levels of OsNAS2 in UM-12115T-1 and the difficulty of purifying sufficient amounts from the plants.

The *E. coli*-derived protein comprised the full-length OsNAS2 sequence fused to a Glycine-Serine-Glycine linker sequence and C-terminal myc-(His)₈ tag and was purified using metal affinity and size exclusion chromatography.

To confirm the microbially expressed OsNAS2 is a suitable surrogate for OsNAS2 expressed in UM-12115T-1 a number of analyses were done to establish their equivalence.

Molecular weight. SDS-PAGE analysis showed that *E. coli*-derived OsNAS2 migrated as a single band with an apparent molecular weight of approximately 37.1 kDa. This is consistent with the predicted molecular weight of native OsNAS2 (34.5 kDa) plus the additional mass contributed by the purification tag.

Peptide mapping. Following digestion with trypsin or chymotrypsin, LC-MS/MS analysis of *E. coli*-derived OsNAS2 identified peptides covering 100% of the expected amino acid sequence. This peptide profile was consistent with that obtained for OsNAS2 detected in UM-12115T-1 grain (section 4.1.1).

N-terminal sequencing. Edman sequencing confirmed the expected N-terminal sequence (MEAQNQEVA) of the *E. coli*-derived OsNAS2.

Functional activity. In an assay measuring the conversion of S-adenosyl methionine to NA, *E. coli*-derived OsNAS2 demonstrated the expected enzymatic activity. The details of this assay are deemed confidential commercial information (CCI) under section 114 of the *Food Standards Australia New Zealand Act 1991* (the FSANZ Act) and can only be disclosed under certain circumstances in accordance with the Act. This information has been evaluated by FSANZ but cannot be disclosed in this report.

Taken together, these results demonstrate *E. coli*-derived OsNAS2 protein possesses the expected physicochemical properties and biological activity of OsNAS2. It can be concluded that *E. coli*-derived OsNAS2 is a suitable surrogate for plant-derived OsNAS2 in the digestibility and heat-stability studies described in section 4.1.3.

4.1.3 Safety of OsNAS2

Bioinformatic analyses

Rice (*Oryza sativa*), the source of the OsNAS2 gene, is recognised as an allergenic food for a small proportion of the population. Bioinformatic analyses, as described in section 3.4.4, were done to compare the sequence of the OsNAS2 protein to known allergenic proteins in the AllergenOnline database (v24), which included known rice allergens.

The search produced a single 8 amino acid exact match (GLAGARGA) against a collagen precursor from *Bos taurus*. Given the glycine-rich nature of this sequence, and the fact that bovine collagen is not considered allergenic, this match is not indicative of an allergenic concern. Full-length and 80-amino acid sliding window searches did not identify any known allergens with homology to OsNAS2.

In addition, OsNAS2 was screened against a curated database of 1,041 coeliac disease-associated peptides and 76 coeliac-inducing proteins using the AllergenOnline Celiac Disease Novel protein risk assessment tool. No matches were identified.

The OsNAS2 amino acid sequence was also compared *in silico* to a database of proteins identified as 'toxins' as described in section 3.4.4. A BLASTP algorithm was used with a BLOSUM62 scoring matrix and an E-value threshold of 0.0001. The search did not identify any known toxins with homology to OsNAS2.

Susceptibility to digestion

E. coli-derived OsNAS2 was incubated at 37°C in simulated gastric fluid (SGF) containing pepsin at an acidic pH of 1.2 (Thomas et al. 2004). Bovine serum albumin (BSA) and β -lactoglobulin were used as digestion-labile and digestion-stable controls, respectively, and were incubated under the same conditions as OsNAS2. Incubation was for 60 minutes, with the extent of digestion over time monitored by SDS-PAGE and western blot.

No intact full-length OsNAS2 was detectable after 0.5 minutes of incubation. Intact BSA control protein was not detectable after 1 minute, while the β -lactoglobulin control remained intact in the reaction mixture after 60 minutes. These results indicate that OsNAS2 is rapidly digested by pepsin.

Heat stability

The heat stability of OsNAS2 was assessed by measuring residual enzymatic activity following heat treatment. *E. coli*-derived OsNAS2 was incubated at temperatures ranging from 25°C to 65°C for 15 minutes, after which its ability to convert S-adenosyl methionine to NA was quantified by LC-MS/MS. Enzymatic activity decreased by approximately 50% following incubation at 40°C, and was undetectable following the 60°C treatment. These results demonstrate that OsNAS2 is thermolabile and is inactivated by temperatures between 40 and 60°C.

Post-translational modifications

Due to the low expression levels, post-translational modification of OsNAS2 could not be evaluated experimentally. Instead, 6 bioinformatic algorithms were used to predict its intracellular localisation and potential for post-translational modification (Figure 6).

The analyses predicted that OsNAS2 lacks signal peptides and organelle-targeting

sequences, consistent with its role as a cytosolic enzyme. While several theoretical glycosylation motifs were identified, glycosylation of OsNAS2 *in planta* is unlikely as the expected location of OsNAS2 in the cytosol means it would not be exposed to glycosylation machinery, which in plants is primarily restricted to the endoplasmic reticulum and Golgi apparatus (Strasser 2016).

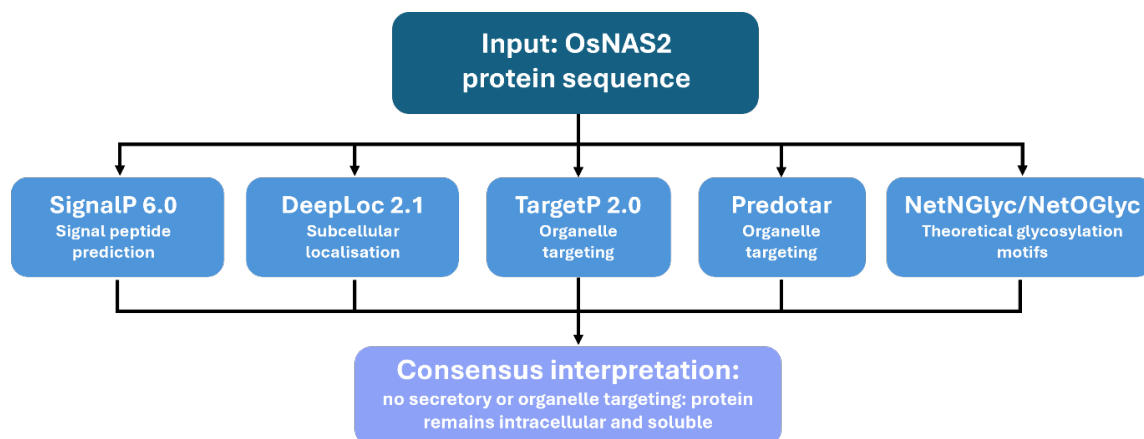


Figure 6. The bioinformatic approach used to assess the potential for post-translational modification of OsNAS2. Multiple algorithms were used to predict the presence of signal peptides, organelle-targeting sequences and glycosylation motifs. The integrated predictions indicated that OsNAS2 lacks signals for secretory pathway or organelle-targeting and is therefore likely to remain in the cytosol.

4.1.4 Conclusion

The OsNAS2 protein is derived from rice, and along with other similar NAS proteins found in cereal crops, has a long history of consumption in many commonly consumed foods. Expression of OsNAS2 in the edible portion of UM-12115T-1 wheat was low (0.024 ng/mg extractable protein). A range of characterisation studies on *E. coli*-derived OsNAS2 confirmed it was a suitable surrogate for use in digestibility and heat stability studies. OsNAS2 was rapidly digested by pepsin and was heat labile at temperatures between 40°C and 60°C. Bioinformatic analyses showed OsNAS2 does not share any meaningful homology with known allergens, coeliac disease-associated proteins, or toxins and is unlikely to undergo post translational glycosylation due to its location in the cytosol. Taken together this indicates the OsNAS2 protein is unlikely to be toxic or allergenic to humans.

4.2 HPT

The *hph* gene (also referred to as *hpt* or *aph(4)-1a*) encodes hygromycin B phosphotransferase (HPT), an enzyme of the aminoglycoside phosphotransferase superfamily that confers resistance to the antibiotic hygromycin B (Rao et al. 1983). In plants, expression of HPT allows for selection of transformants by allowing their growth on hygromycin (Miki and McHugh 2004; Stogios et al. 2011).

Hygromycin B is an aminoglycoside antibiotic produced by *Streptomyces hygroscopicus* that inhibits protein synthesis in both prokaryotic and eukaryotic cells by binding to the A-site of the ribosome (Eustice and Wilhelm 1984). HPT catalyses the ATP-dependent phosphorylation of hygromycin B at its 4-hydroxyl group, blocking the antibiotic from binding the ribosomal A-site and therefore preventing it from inhibiting translation (Rao et al. 1983; Stogios et al, 2011).

Because its substrate binding pocket is highly specific to hygromycin B's four-ring structure,

HPT sterically excludes other aminoglycosides. As such, it does not confer resistance to other antibiotics such as kanamycin or gentamycin (Stogios et al. 2011; EFSA 2004). HPT has no known endogenous plant substrates and is biologically inert in the absence of hygromycin B (Miki and McHugh 2004).

While the *hph* gene and its encoded protein are present in event UM-12115T-1, its function is limited to the initial transformation stage (section 3.1) and it has no function in the final wheat lines.

4.2.1 Expression and characterisation of HPT in wheat lines containing event UM-12115T-1

In UM-12115T-1, the *hph* expression cassette encodes a protein of 341 amino acids with a molecular weight of ~38 kDa. The protein sequence is identical to the HPT protein expressed in cotton line COT102 (Application A509; FSANZ 2004). The protein is also identical in sequence to the HPT protein assessed in published safety studies (Lu et al. 2007).

Expression of the introduced HPT protein was characterised in both grain (flour) and leaf tissue of UM-12115T-1 using targeted LC-MS/MS. HPT was not detected in either the flour or leaf samples. Despite this, the expression of HPT in regenerating cells following transformation was sufficient to provide the cells with hygromycin tolerance for the purpose of selecting transgenic transformants, demonstrating that the HPT protein is functional (see section 3.1).

4.2.2 Safety of HPT

The safety of HPT has been considered in 2 previous FSANZ assessments.⁷ For these assessments, studies on potential allergenicity and toxicity were submitted and assessed. These previous assessments did not raise any safety concerns.

The *hph* gene has been widely used as a selectable marker gene for development of GM plants (Miki and McHugh 2004). Published literature also supports the safety of the HPT protein (Lu et al. 2007; Zhuo et al. 2009). Additionally, the safety of HPT has been evaluated by others, who concluded that using HPT as a selectable marker in GM plants does not pose a risk to human or animal health or the environment (EFSA 2004; EFSA 2009; OGTR 2024).

Since the sequence of the HPT protein expressed in UM-12115T-1 wheat lines is identical to a previous HPT sequence assessed by FSANZ (in Application 509), no further safety evaluation is required other than examination of updated bioinformatics searches.

The applicant submitted updated bioinformatic studies for HPT that looked for amino acid sequence similarity to known protein allergens and toxins (April 2026). In addition, the applicant conducted bioinformatic analyses to predict the likelihood of post-translational modifications (see section 4.1.3). FSANZ has assessed the data submitted by the applicant and the results do not alter conclusions reached in previous assessments.

4.2.3 Conclusion

HPT expressed in event UM-12115T-1 is identical to a previously assessed HPT protein and is undetectable by LC-MS/MS in either flour or leaf tissue. Despite this, HPT is functional, as demonstrated by its ability to provide plant cells with hygromycin tolerance following

⁷ Cotton line COT102 (Application A509; FSANZ 2004) and safflower lines 26 and 40 (Application A1156; FSANZ 2018).

transformation. Updated bioinformatic analyses confirm that HPT has no amino acid sequence similarity to known allergens or toxins.

5 Compositional analyses

The main purpose of compositional analyses is to determine if the genetic modification has resulted in any unexpected compositional change to the food. Compositional analyses are also important for evaluating the intended effect where there has been a deliberate change to the composition of the food.

The aim of the compositional analysis is to measure 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 GM and conventional wheat are outlined in the OECD Consensus Document on Compositional Considerations for New Varieties of Wheat (OECD 2003) and include proximates (protein, fat, fibre, ash, carbohydrates), amino acids, fatty acids, minerals, vitamins and the anti-nutrient phytic acid (Figure 7).

Information was also provided on the NA content of UM-12115T-1 wheat lines in comparison with a non-GM isogenic control and additional conventional reference wheat lines (see section 5.4).

5.2 Study design

Wheat event UM-12115T-1, a near-isogenic non-GM control and 2 commercial reference varieties (Sunmaster and Ironbark) were grown and harvested from 4 field trial sites in New South Wales, Australia during the 2025 growing season.⁸ Grain was harvested at maturity, and compositional analyses were performed by an accredited third-party laboratory.

A total of 68 analytes in grain were assessed (see Figure 8 for a complete list). Pooled data from the 4 sites were analysed using a linear mixed-effects model, with genotype included as a fixed effect, and site and replicate included as random effects. For each analyte, 'descriptive statistics' (mean, standard deviation and range) were generated.

Pairwise comparisons between UM-12115T-1 and the non-GM control were conducted using Dunnett's test at a significance level of 0.05. Any statistically significant differences were evaluated further to assess whether they were likely to be biologically meaningful.

In addition to statistical comparisons with the control, analyte values were compared with those of the 2 commercial reference varieties, as well as literature ranges compiled from publicly available data (Obert et al. 2004; Heuzé et al. 2015; OECD 2003). This takes natural

⁸ The location of the 4 field trial sites: Moma, Leeton 1, Leeton 2 and Binya.

variability into account in non-GM wheat varieties across a wider range of agronomic and environmental conditions, as well as different genetic backgrounds.

Key analytes (proximates and minerals) were also measured in forage, but the results are not included in this report. It is noted however that, in the combined site analysis, the analyte levels in UM-12115T-1 forage did not differ significantly from those of the control.

Proximates and fibre (8) Energy Carbohydrates Protein Ash Total free sugars Total dietary fibre Fat Gluten	Amino acids (18) Aspartic acid Threonine Isoleucine Serine Alanine Leucine Glutamic acid Proline Phenylalanine Glycine Tyrosine Methionine Histidine Valine Hydroxyproline Arginine Lysine Taurine	Minerals (12) Calcium Sodium Copper Zinc Iron Arsenic Magnesium Cadmium Manganese Lead Phosphorus Nickel
Fatty acids (24) Myristic acid Arachidic acid Omega-9 fatty acids Palmitic acid Eicosonic acid α-Linolenic acid (AHA) Palmitoleic acid Eicosadienoic acid Eicosapentaenoic acid (EPA) Heptadecanoic acid Behenic acid Docosahexaenoic acid (DHA) Stearic acid Erucic acid Saturated fatty acids Oleic acid Lignoceric acid Monounsaturated fatty acids Linoleic acid Omega-3 fatty acids Polyunsaturated fatty acids α-Linoleic acid Omega-6 fatty acids Trans fatty acids		Vitamins and anti-nutrients (6) Thiamine (vitamin B1) Riboflavin (vitamin B2) Niacin (vitamin B3) Pyridoxine (vitamin B3) Folate Phytate

Figure 7. Analytes measured in the combined multi-site compositional analysis of UM-12115T-1 wheat grain.

5.3 Analyses of key components in grain

Of the 68 analytes for which mean values were provided, there were 3 for which there was a statistically significant difference ($p < 0.05$) between UM-12115T-1 and the non-GM control: thiamine, histidine and phenylalanine. A summary of these 3 analytes is provided in Figure 8. For iron (Fe) and zinc (Zn), statistically significant increases were seen in UM-12115T-1 when compared to the Sunmaster and Ironbark varieties, but there was no significant difference in the levels of Fe or Zn between UM-12115T-1 and the near-isogenic non-GM control. For the complete data set, including values for the analytes for which no statistically significant differences were found, refer to the [Application dossier](#)⁹ (pages 76 - 88).

For all 3 analytes where a statistically significant difference was found, the UM-12115T-1 mean was nested within the control range (Figure 8b-d), indicating the genetic modification of UM-12115T-1 has a smaller impact on the levels of these analytes than does natural variation within the conventional control. Furthermore, the observed UM-12115T-1 means fell well within the natural variability seen in the publicly available data (pale gold bars, Figure 9), and within the ranges observed for the Sunmaster and Ironbark reference varieties (grey bars, Figure 8).

For histidine (Figure 8c), the lower end of the range observed for UM-12115T extended below the publicly available reference range. To determine whether this difference was biologically meaningful, additional compositional data from grain harvested from the 2022

⁹ The Application dossier can be found on the A1356 webpage – <https://www.foodstandards.gov.au/food-standards-code/applications/a1356-food-derived-increased-nicotianamine-wheat-lines-containing>.

and 2023 growing seasons was considered. In both years, histidine values for UM-12115T-1 were nested within the publicly available range. In addition, Near-Infrared Reflectance Spectroscopy was used to predict the amino acid profile of UM-12115T-1 wheat as well as the non-GM control and other reference lines. For histidine, the minimum and maximum range for UM-12115T-1 closely matched the biological variance observed across the non-GM commercial reference lines. Collectively, these data indicate the observed variation in histidine is attributable to variations in agronomic and environmental conditions, rather than a biologically meaningful difference in the GM event.

The differences reported here are therefore consistent with the normal biological variability that exists in wheat.

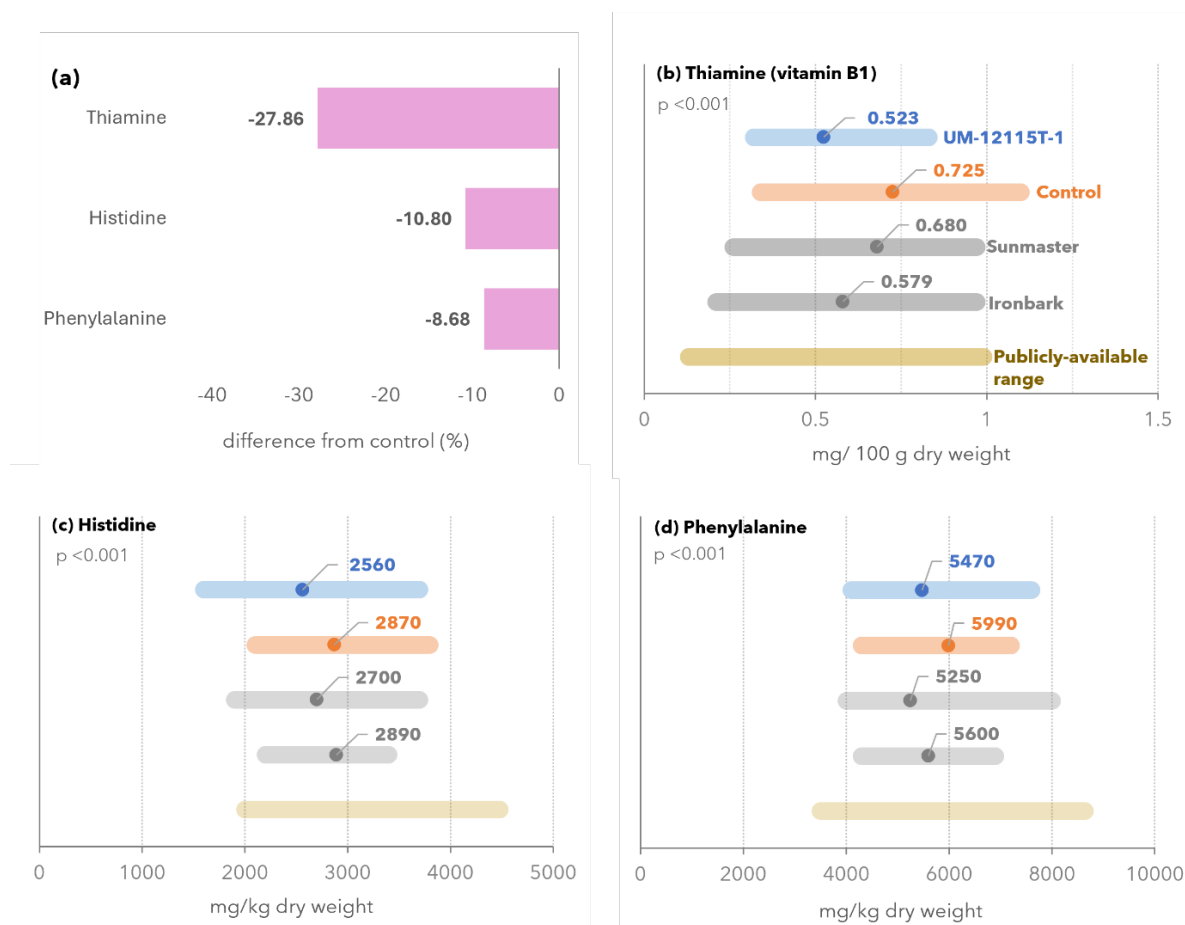


Figure 8. Visual summary of statistically significant compositional differences between UM-12115T-1 wheat and the conventional control wheat. **(a)** Deviation of the mean UM-12115T-1 value from the mean control value for each of the 3 analytes for which a statistically significant difference was found, expressed as a percentage of the mean control value. **(b) – (d)** Measured means (dots) and ranges (coloured bars) for UM-12115T-1 (blue) and the conventional control (orange) for the 3 analytes as labelled. The means and ranges of the reference wheat varieties Sunmaster and Ironbark are shown in grey. The light gold bars represent the consolidated range of values from the literature and publicly available databases. Note that the x-axes vary in scale and unit for each component.

5.4 Targeted quantification of nicotianamine in grain

The expression of OsNAS2 in event UM-12115T-1 is expected to increase NA concentrations relative to the non-GM comparator, as it is the primary metabolite arising from the enzyme reaction catalysed by OsNAS2 (see section 4.1 and Figure 5). To assess this

effect, NA concentrations were measured in grain from UM-12115T-1, the near-isogenic non-GM control, and 5 conventional reference wheat varieties across multiple field sites. Details of these analyses are CCI; the key findings are summarised below.

Analysis of grain collected from a single field site over 3 consecutive growing seasons (2021-2023) showed that environmental conditions significantly influenced absolute NA concentrations. However, UM-12115T-1 consistently contained significantly higher NA levels than the non-GM comparator in each year examined.

To further assess trait stability across a wider range of environmental conditions, NA concentration data from 4 field trials across different locations and growing seasons were pooled. Across the pooled dataset, UM-12115T-1 consistently showed elevated NA concentrations relative to the corresponding non-GM comparator across all environments.

Comparison with a set of 5 conventional reference wheat varieties showed that, although UM-12115T-1 consistently contained higher NA concentrations compared to the non-GM comparator, NA concentrations remained within the range observed among conventional wheat varieties.

5.5 Conclusion

The compositional data indicate there are no biologically meaningful differences in the levels of key constituents in UM-12115T-1 wheat lines when compared with conventional non-GM wheat varieties already available in agricultural markets.

While the NA concentration in UM-12115T-1 grain was significantly increased compared to the non-GM comparator, its level remained within the range of natural variation observed in conventional wheat varieties. No significant differences in grain iron or zinc levels were observed between UM-12115T-1 and the non-GM control. Grain from wheat lines containing event UM-12115T-1 is therefore compositionally equivalent to grain from conventional wheat varieties.

6 Nutritional impact

In most cases, an assessment of nutritional impact 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.

If the compositional analyses identify biologically meaningful changes in the levels of certain nutrients in the GM food, additional nutritional assessment may assist with assessing the consequences of the changes and determine whether nutrient intakes are likely to be altered by the introduction of such foods into the food supply. Evidence indicates that feeding studies using target livestock species will add little to the safety assessment (OECD 2003; Bartholomaeus et al. 2013; Herman and Ekmay 2014).

The compositional analyses demonstrated that wheat lines containing event UM-12115T-1 are compositionally equivalent to the non-GM comparator, except for the intended increase in NA concentration.

Although UM-12115T-1 grain contains increased NA concentrations relative to the non-GM comparator, the concentrations remain within the range observed among conventional wheat varieties (section 5.4). In addition, published studies report substantially higher NA concentrations in a range of commonly consumed plant foods, including legumes and

vegetables, than in cereal grains such as wheat, rice and maize (Izawa et al. 2008; Izawa and Aoyagi 2012; Yamaguchi and Uchida 2012).

The applicant also provided evidence from published studies indicating that increased grain NA concentrations can improve the bioavailability of Fe and Zn in wheat (Beasley et al. 2019a; Beasley et al. 2019b; Beasley et al. 2022). However, compositional analyses of UM-12115T-1 showed no significant differences in total grain iron or zinc concentrations compared with the non-GM control. Any increases in bioavailability resulting from the increase in NA UM-12115T-1 would not be expected to materially affect population nutrient intakes given much higher NA levels in other wheat varieties and commonly consumed plant foods.

The introduction of food derived from UM-12115T-1 wheat into the food supply is therefore expected to have negligible nutritional impact.

7 Other information

The applicant supplied a summary of 2 poultry feeding studies conducted to evaluate the nutritional efficacy of wheat event UM-12115T-1. Such feeding studies are not an Application Handbook requirement, nor considered by FSANZ to be necessary for safety assessment purposes consistent with international regulatory data requirements. However, they are summarised below as additional supporting information.

In both trials, UM-12115T-1 wheat was incorporated into broiler diets and evaluated against a conventional control (Australian Standard White) and the near-isogenic non-GM control wheat. The results of the studies showed that, under the study conditions, birds fed UM-12115T-1 wheat did not exhibit significantly different weights or feed conversion ratios compared to the non-GM comparator over a 42-day period.

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Appendix 1

Genetic elements in pUbi-OsNAS2

Genetic Element	Source	Relative position	Size (bp)	Description/Function
Right Border (RB)	Synthetic	1-25	25	Secondary cleavage site releases ssDNA inserts from pMDC32
Intervening sequence	Synthetic	26-259	234	Used for DNA cloning
OsNAS2 expression cassette				
Ubi1 Promoter	<i>Zea mays</i>	260-1767	1508	Drives expression of the OsNAS2 gene
Intervening sequence	Synthetic	1768-1864	97	Used for DNA cloning
OsNAS2	<i>Oryza sativa</i>	1865-2845	981	Coding sequence for the OsNAS2 protein, a key enzyme in the biosynthesis of nicotianamine
Intervening sequence	Synthetic	2846-2958	113	Used for DNA cloning
Nopaline synthase gene terminator (<i>nos</i>)	<i>Agrobacterium tumefaciens</i>	2959-3206	248	3' non-translated region of the nopaline synthase gene; terminator and polyadenylation signal for the OsNAS2 gene transcript
Intervening sequence	Synthetic	3207-3526	320	Used for DNA cloning
hph expression cassette				
Enhanced CaMV35S promoter	Cauliflower mosaic virus	3527-4204	678	Drives expression of the <i>hph</i> gene
Intervening sequence	Synthetic	4205-4271	67	Used for DNA cloning
Hygromycin resistance gene (<i>hph</i>)	<i>Klebsiella sp.</i> via <i>E. coli</i>	4272-5294	1023	Coding sequence for hygromycin phosphotransferase (HPT), an antibiotic resistance gene and selectable marker
Intervening sequence	Synthetic	5298-5336	39	Used for DNA cloning
CaMV35S Poly A	Cauliflower mosaic virus	5337-5511	175	Terminator for <i>hph</i> gene transcript
Intervening sequence	Synthetic	5512-5588	77	Sequence used for DNA cloning
Left border (LB)	Synthetic	5589-5613	25	Secondary cleavage site releases ssDNA inserts from pMDC32
Intervening sequence	Synthetic	5614-6037	424	Used for DNA cloning
Kanamycin resistance gene (<i>nptIII</i>)	<i>Enterococcus faecalis</i> pJH1	6038-6832	795	Kanamycin resistance (<i>aphIII/nptIII</i> 3'5"-aminoglycoside phosphotransferase type III from <i>Enterococcus faecalis</i> pJH1)
Intervening sequence	Synthetic	6833-7122	290	Used for DNA cloning
Origin of replication for pBR322 (pBR322 <i>ori</i>)	Synthetic	7123-7403	281	Origin of replication
Intervening sequence	Synthetic	7404-7692	289	Used for DNA cloning
bom	Synthetic	7693-7833	141	Basis of mobility region from pBR322
Intervening sequence	Synthetic	7834-8212	379	Used for DNA cloning
Intervening sequence pVS1	<i>Pseudomonas fluorescens</i> pVS1	8213-9213	1001	Origin of replication for the <i>Pseudomonas</i> plasmid pVS1
Intervening sequence	Synthetic	9214-11866	2653	Used for DNA cloning