

13 November 2018

[63-18]

Supporting document 1

Safety Assessment Report – Application A1156 (at Approval)

Food derived from Super High Oleic Safflower Lines 26 and 40

Summary and conclusions

Background

GO Resources Pty Ltd has submitted an application to FSANZ to vary Schedule 26 in the *Australia New Zealand Food Standards Code* (the Code) to include food from either of two lines of genetically modified (GM) super high oleic (SHO) safflower (*Carthamus tinctorius*) (herein referred to as SHO safflower). The two lines have Organisation for Economic Co-operation and Development (OECD) Unique Identifiers GOR-73226-6 and GOR-73240-2 and will be referred to as SHO26 and SHO40 respectively. The lines have been genetically modified to increase the proportion of oleic acid (18:1) produced in the seed oil from around 75% to around 92%, with concomitant reduction in linoleic acid (18:2) from approximately 15% to 2%, and palmitic acid (16:0) from approximately 6% to 3%.

The genetic modification uses RNA interference (RNAi) to suppress the expression of two native safflower genes involved in fatty acid synthesis – the palmitoyl-ACP thioesterase (*CtFATB*) gene and the $\Delta 12$ desaturase (*CtFAD2-2*) gene. Fragments of these genes, derived from safflower, have been introduced and their transcription results in the formation of a hairpin structure comprising double-stranded RNA (dsRNA) which is processed by the endogenous cellular machinery of the host into short interfering RNAs (siRNAs). In turn, these siRNAs then direct the degradation of the messenger RNA (mRNA) transcribed from the host endogenous genes, suppressing its translation into proteins. The result of suppressing expression of the genes is that the proportion of oleic acid in the safflower seed oil is increased.

SHO26 and SHO40 also contain the hygromycin resistance gene, *hph*, expressing the enzyme hygromycin B phosphotransferase (HPH), which confers resistance to the antibiotic hygromycin. The gene is derived from a plasmid from the common bacterium *Escherichia coli*. It was used as a selectable marker to assist with identification of transformed safflower cells in the early stages of selection.

This safety assessment report addresses only food safety and nutritional issues of the GM food *per se*. It therefore does not address:

- environmental risks related to the environmental release of GM plants used in food production
- the safety of animal feed, or animals fed with feed, derived from GM plants

- the safety of food derived from the non-GM (conventional) plant.

History of use

Cultivated safflower (*Carthamus tinctorius*; family Asteraceae) has been utilised by man for several thousand years. While probably grown originally for its red and yellow pigments, obtained from the petals of the flowers, the seed oil is now the most commercially-sourced product.

Two types of safflower oil are commercially available: one high in the monounsaturated fatty acid oleic acid (75% minimum) and the other high in the polyunsaturated fatty acid linoleic acid (75% minimum). Both oil types are used in the food industry as well as in the manufacture of pharmaceuticals, cosmetics, paint and varnish products. In the food industry the high linoleic acid oil (which contains higher linoleic acid than other oils such as corn, soybean, cottonseed, peanut or olive oils), is suited to salad oils and soft margarines while the high oleic oil is useful for frying, as oleic acid confers stability under high heat conditions and therefore extends the fry life of an oil. The meal is not suitable for human consumption because of anti-nutritional factors. However, protein isolate from the seeds has potential for use in food.

Molecular characterisation

The two SHO safflower lines were generated through *Agrobacterium*-mediated transformation with a single T-DNA containing two expression cassettes. Comprehensive molecular analyses indicate in both lines there is one insertion site containing a single, intact copy of the T-DNA. The introduced genetic material is stably inherited across multiple generations and in different genetic backgrounds. No antibiotic resistant marker from the plasmid backbone has been incorporated into the transgenic locus of either line. A small, non-functional sequence of the plasmid backbone from one of the origins of replication has been incorporated into the junction region of SHO26 but does not raise any safety concerns.

Several approaches were taken to analyse the expression of the two genes targeted for silencing. Transcript analysis showed a significant reduction in expression of mRNA from both the *CtFAD2-2* and *CtFATB* genes in SHO26 and SHO40. Lipid analysis indicated down-regulation was confined to tissue developmentally derived from the seed.

The only siRNAs that were generated in both SHO26 and SHO40 mapped against both *CtFAD2.2* and *CtFATB* precisely within the confines delineated by the hairpin structure of the introduced fragments, thereby confirming that the silencing observed in SHO26 and SHO40 is indeed mediated by the formation of the expected siRNAs and not by some other component associated with the hairpin structure.

Characterisation and safety assessment of new substances

SHO26 and SHO40 express one novel protein – HPH. This protein was detectable at low level in leaf and seed tissue. Previous safety assessment of HPH, along with evidence from the literature, indicates the protein would be rapidly degraded in the digestive system following ingestion and would be inactivated by heating. Updated bioinformatic studies considered in this assessment confirm the lack of any significant amino acid sequence similarity to known protein toxins or allergens.

There are no concerns with the safety of the dsRNA or subsequent siRNAs produced in SHO26 and SHO40.

Compositional analyses

Detailed compositional analyses were conducted on seed from the two SHO safflower lines grown in two different locations in Australia. The seeds were analysed for proximates, amino acids, fatty acids, minerals, vitamins and anti-nutrients. The levels of each analyte in SHO26 and SHO40 were compared to levels in: a) the non-GM parental line, M1582; b) two or three non-GM commercial reference lines grown at the same locations; and c) levels recorded in the literature.

A total of 48 individual analytes (plus energy) and four fatty acid groupings were analysed. Of the individuals, six had a high number of the observations below the limit of detection and were excluded from the statistical analysis. The data for 42 individual analytes (plus energy) were therefore considered statistically. Of these 42 individual analytes, only three, all fatty acids, differed significantly between SHO26, SHO40 and M1582. The changes in fatty acid profile were consistent with those expected as a result of the introduction of the RNAi cassette.

Apart from the intended change to fatty acid profile, seed from SHO26 and SHO40 is otherwise compositionally equivalent to seed from conventional safflower varieties.

Nutritional impact

While the levels of oleic and linoleic acids have been altered in SHO26 and SHO40, the total percentage of unsaturated fatty acids (monounsaturated + polyunsaturated) is approximately the same in the two SHO safflower lines as that in currently available non-GM high oleic safflower lines.

A dietary intake assessment was conducted by FSANZ that considered the dietary intake of oleic acid from the current food supply (baseline intake) and two scenarios to account for potential additional intake of oleic acid due to the introduction of SHO safflower to the Australian and New Zealand food supplies. The modelling indicated the addition of SHO safflower oil would make little to no difference to oleic acid intakes. Given this, and the fact that oils are not the major source of linoleic acid in the diet, the introduction of SHO safflower oil to the food supply is unlikely to decrease linoleic acid intake outside of normal daily variation in intakes. It is concluded that consumption of SHO safflower will not pose a nutritional concern to the Australian and New Zealand populations.

Conclusion

No potential public health and safety concerns have been identified in the assessment of SHO26 and SHO40. On the basis of the data provided in the present application, and other available information, food derived from SHO26 or SHO40 is considered to be as safe for human consumption as food derived from conventional safflower varieties.

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List of Abbreviations

ABS	Australian Bureau of Statistics
ACP	acyl carrier protein
AI	Adequate Intake
HPH	Aminoglycoside phosphotransferase (same as HPH)
BLASTn	Basic Local Alignment Search Tool: nucleotide
bp	base pairs
CaMV	Cauliflower mosaic virus
CBI	Crop Biofactories Initiative
cDNA	copy DNA
CLIP	closed-loop identity preserved
Code	<i>Australia New Zealand Food Standards Code</i>
CSIRO	Commonwealth Scientific and Research Organisation
DNA	deoxyribonucleic acid
dsRNA	double-stranded RNA
dw	dry weight
EAR	estimated average requirement
FAD	fatty acid desaturase
FAO	Food & Agricultural Organization of the United Nations
FASTA	Fast Alignment Search Tool – All
FAT	fatty acid acyl transferase
FSANZ	Food Standards Australia New Zealand
fw	fresh weight
g	gram
GM	genetically modified
HPH	Hygromycin phosphotransferase
IS	intervening sequence(s)
kDa	kilo Dalton
kg	kilogram
LB	Left Border of T-DNA (<i>Agrobacterium tumefaciens</i>)
LOD	Limit of Detection
mg	milligram
mRNA	messenger RNA
NCBI	National Centre for Biotechnology Information
NNPAS	Australian National Nutrition and Physical Activity Survey
NNS	Australian National Nutrition Survey
nt	nucleotide
NZ	New Zealand
OECD	Organisation for Economic Co-operation and Development
OGTR	Office of the Gene Technology Regulator
ORF	open reading frame
PCR	polymerase chain reaction
RB	Right Border of T-DNA (<i>Agrobacterium tumefaciens</i>)
RNA	ribonucleic acid
RNAi	RNA interference
SHO	Super high oleic
siRNA	short interfering RNA
T	tonnes
TAGs	triacylglycerols
T-DNA	transfer DNA
Ti	tumour inducing
U.S.	United States of America
USDA	United States Department of Agriculture
UTR	untranslated region

1 Introduction

GO Resources Pty Ltd has submitted an application to FSANZ to vary Schedule 26 in the *Australia New Zealand Food Standards Code* (the Code) to include food from either of two lines of genetically modified (GM) super high oleic (SHO) safflower (*Carthamus tinctorius*) (herein referred to as SHO safflower). The two lines have OECD Unique Identifiers GOR-73226-6 and GOR-73240-2 and will be referred to as SHO26 and SHO40 respectively. The lines have been genetically modified to increase the proportion of oleic acid (18:1) produced in the seed oil from around 75% to around 92%, with concomitant reduction in linoleic acid (18:2) from approximately 15% to 2%, and palmitic acid (16:0) from approximately 6% to 3% (Wood et al. 2013).

The genetic modification uses RNA interference (RNAi) to suppress the expression of two native safflower genes involved in fatty acid synthesis – the palmitoyl-ACP thioesterase (*CtFATB*) gene and the $\Delta 12$ desaturase (*CtFAD2-2*) gene. Fragments of these genes, derived from safflower, have been introduced and their transcription results in the formation of double-stranded RNA (dsRNA) which is processed by the endogenous cellular machinery of the host into short interfering RNAs (siRNAs). In turn, these siRNAs then direct the degradation of the messenger RNA (mRNA) transcribed from the host endogenous genes, thereby suppressing translation into proteins. The result of suppressing the genes is that the proportion of oleic acid in the safflower seed oil is increased.

SHO26 and SHO40 also contain the hygromycin resistance gene, *hph*, expressing the enzyme hygromycin B phosphotransferase (HPH) also known as aminoglycoside phosphotransferase (APH4), which confers resistance to the antibiotic hygromycin. The gene is derived from a plasmid from the common bacterium *Escherichia coli*. It was used as a selectable marker to assist with identification of transformed safflower cells in the early stages of selection. APH4 has been previously assessed by FSANZ in cotton application A509 (FSANZ 2004).

The applicant states the main use of SHO safflower will be for production of oil for use in the lubricant, fine chemical, bioplastics, pharmaceutical and cosmeceutical industries but could also be applicable to the food and personal care industries. The technology will be commercialised within a specialised, 'closed-loop' identity preserved (CLIP) quality assured management program. The oil will be sold to domestic and export market processors, with the meal being directed to use as a stock feed. There is no intention that SHO safflower grain would enter the export or domestic grain markets.

An application for commercial release of the two safflower lines was submitted to the Office of the Gene Technology Regulator (OGTR) ([DIR 158](http://www.ogtr.gov.au/internet/ogtr/publishing.nsf/Content/dir158)¹) and a licence was issued in June 2018. It is possible that, in the future, the Applicant may seek regulatory approval for environmental release in the U.S. It is therefore anticipated food products derived from SHO safflower will enter the Australian and New Zealand food supplies mainly through local production with possible future supplementation from imports.

¹ <http://www.ogtr.gov.au/internet/ogtr/publishing.nsf/Content/dir158>

2 History of use

2.1 Host and donor organism

Cultivated safflower (*Carthamus tinctorius*; family Asteraceae) has been utilised by man for several thousand years (Smith 1996) and is believed to have had a single origin of domestication some 4,000 years ago in a region known as the Fertile Crescent (an area of the Middle East stretching in an arc from the Nile to the Tigris and Euphrates rivers). While probably grown originally for its red and yellow pigments, obtained from the petals of the flowers, the seed oil is now the most commercially-sourced product.

The original cultivars of safflower contained high levels of linoleic acid, but a high oleic natural variant was described and then bred in the 1960s (Horowitz and Winter 1957; Knowles and Mutwakil 1963; Knowles et al. 1965) and high oleic safflower varieties were subsequently commercialised. This means there are now two types of safflower that produce different kinds of oil: one high in the monounsaturated fatty acid oleic acid (75% minimum) and the other high in the polyunsaturated fatty acid linoleic acid (75% minimum) (AOF 2015). Both oil types are used in the food industry as well as in the manufacture of pharmaceuticals, cosmetics, paint and varnish products (Mündel et al. 2004; Popov and Kang 2011). In the food industry the high linoleic acid oil (which contains higher linoleic acid than other oils such as corn, soybean, cottonseed, peanut or olive oils), is suited to salad oils and soft margarines while the high oleic oil is useful for frying, as oleic acid confers greater stability under high heat conditions and therefore extends the fry life of an oil. Oils high in monounsaturated fatty acids also have a better shelf life. The neutral flavour and odour of high oleic safflower oil also allows for a wide range of other food applications, including: spray oil for dried fruits, liquid flavour dispersant, seasonings and nutritional supplements (Oilseeds International Ltd 2016).

The meal, left after oil extraction, is a high quality protein supplement (around 24% protein) for use in livestock feed but is not suitable for human consumption because of anti-nutritional factors (Salunkhe et al. 1992 and also see discussion in Section 5.2.7). However, protein isolate from the seeds has potential for use in food (Zayas 1997; Ulloa et al. 2011).

Safflower is a minor oilseed crop globally (FAOSTAT 2017). In 2015-2016 safflower seed and oil trade were negligible in Australia (ABARES 2017). The current area in Australia planted with safflower is around 10,000 ha although in 1979 it peaked at 74,688 ha (GRDC 2010). The main safflower cultivars developed in Australia were Sironaria and Sirothora, both released by the Commonwealth Scientific and Research Organisation (CSIRO) in 1986 (Harrigan 1987) and both linoleic oil types. Sironaria has become the most widely grown cultivar in Australia. The CSIRO breeding programme ceased and there have been no further safflower breeding programmes in Australia although a few cultivars have been imported from overseas. New Zealand neither grows nor trades significant quantities of safflower.

The variety used as the host for the transformation process generating SHO safflower was the advanced breeding Mexican line, M1582. This high oleic acid line was imported into Australia from a Mexican breeding program. The breeding line has been field evaluated since 2012 under the genotype code CC1582-1-2 (see e.g. Hertel 2016). It has also been referred to as line CBIY (Crop Biofactories Initiative-Yellow) in reference to the flower colour.

2.2 Other donor organisms

2.2.1 *Escherichia coli*

The bacterium *Escherichia coli* is the likely source of the selectable marker gene, *hph*. *E. coli*

belongs to the Enterobacteriaceae family, a relatively homogeneous group of rod-shaped, Gram-negative, facultative aerobic bacteria. Members of the genus *Escherichia* are ubiquitous in the environment and are normally found in the digestive tracts of vertebrates, including humans where they are the most abundant facultative aerobe (Donnenberg 2002). The vast majority of *E. coli* strains are harmless to humans, although some strains can cause diarrhoea and occasionally urinary tract infections.

Some strains of *E. coli*, such as the enterohaemorrhagic *E. coli* group (e.g. O157:H7), are particularly virulent pathogenic strains responsible for causing serious food-borne illness. This particular group of pathogenic *E. coli* are distinct from the strains of *E. coli* (the K-12 strains) that are used routinely in laboratory manipulations; the *E. coli* used as a donor organism in this application is K-12. The K-12 strains of *E. coli* have a long history of safe use and are commonly used as protein production systems in many commercial applications, (Baeshen et al. 2015) including for pharmaceutical products and food ingredients (e.g. Schedule 18 of the Code permits the use of chymosin derived from *E. coli* K-12 strain as a food processing aid).

2.2.2 Regulatory elements from other organisms

Genetic elements from three other organisms not mentioned above - *Ricinus communis* (castor bean), Cauliflower mosaic virus (CaMV) and *Agrobacterium tumefaciens* have been used in the genetic modification of SHO safflower (refer to Table 1). These sequences are used to drive, terminate or enhance expression of the novel genetic material. Neither CaMV nor *A. tumefaciens* is associated with toxic or allergenic responses in humans and the genetic elements derived from them are not pathogenic *per se* and do not cause pathogenic symptoms in SHO safflower. While the ricin protein in beans of *R. communis* is associated with human poisoning (Audi et al. 2005), the *Cat-1* intron sequence (Table 1) from *R. communis*, used in SHO safflower, does not code for a protein and is not part of the full-length ricin gene which is around 1900 bp (Halling et al. 1985).

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
- a characterisation of the dsRNA expressed in SHO safflower.

Unpublished Studies submitted:

- 2018. Molecular characterisation of Event 26 and Event 40. Study Report No. GOR-SHOSO-MOL-18-1.0. GO Resources Pty Ltd
- 2018. Evaluation of allergen and toxin homology of start-to-stop open reading frames in Event 26 and Event 40. Study Report No. GOR-SHOSO-ORF-17-2.0. GO Resources Pty Ltd
- 2018. Assessment of the stability of the T-DNA insert in Event 26 and Event 40. Study report No. GOR-SHOSO-STAB-18-1.0. GO Resources Pty Ltd
- 2018. RNA expression of down-regulated genes in Event 26 and Event 40. Study report No. GOR-SHOSO-EXP-18-1.0. GO Resources Pty Ltd

3.1 Method used in the genetic modification

The method was based on that described by Belide et al (2011). Cotyledons from seedlings of cultivar M1582 were co-cultured with disarmed *Agrobacterium tumefaciens* strain AGL1 (Lazo et al. 1991) harbouring a binary vector system (Deblaere et al. 1987). The transformation vector, plasmid pCW732 (Figure 1), contained the DNA sequences of interest between right border (RB) and left border (LB) in the T-DNA sequence of the binary expression vector pORE-CBIb (Coutu et al. 2007) (Figure 2).

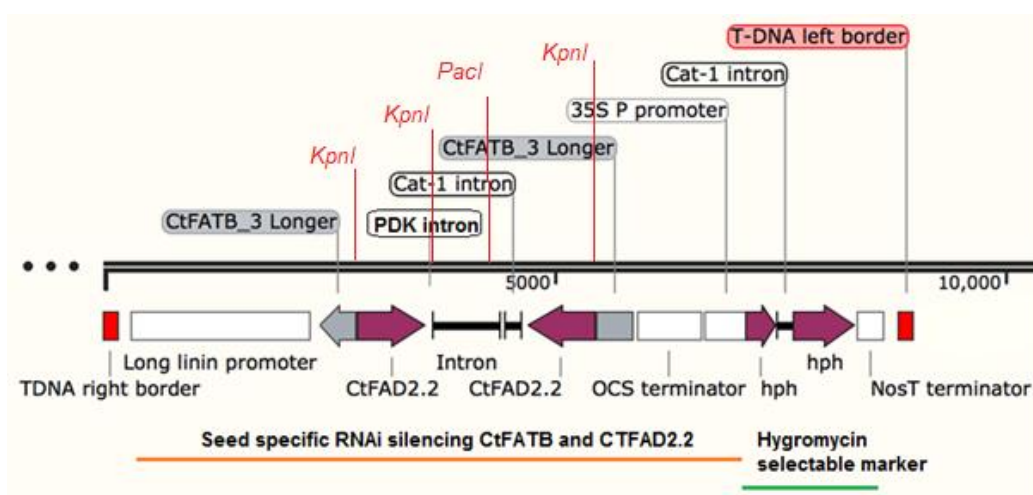


Figure 1: Representation of genes, regulatory elements and restriction enzyme sites (in red) contained in transformation vector pCW732

Following co-culture, cotyledon segments were transferred to a callus initiation medium containing cefotaxime and timentin (to inhibit the growth of excess *Agrobacterium*), and hygromycin (for selection of putative transformants). Surviving calli were transferred to a shoot initiation medium and resulting shoots (T_0) were isolated and transferred to shoot outgrowth then shoot elongation media. Shoots were then grafted onto rootstocks obtained from decapitated seedlings growing in seedling raising mixture. Leaves from successful grafted plants were used for testing of putative transgenic lines by polymerase chain reaction (PCR). Positive plantlets (generation T_0) were transferred to the glasshouse for 3 – 4 months until mature. Fatty acid profiles were non-destructively assessed in seeds from the T_1 and T_2 generations. Southern Blot analyses were used to select those events in which there was only a single copy of the T-DNA. SHO26 and SHO40 were ultimately chosen as the lead events based on superior agronomic, biochemical, genetic and molecular characteristics.

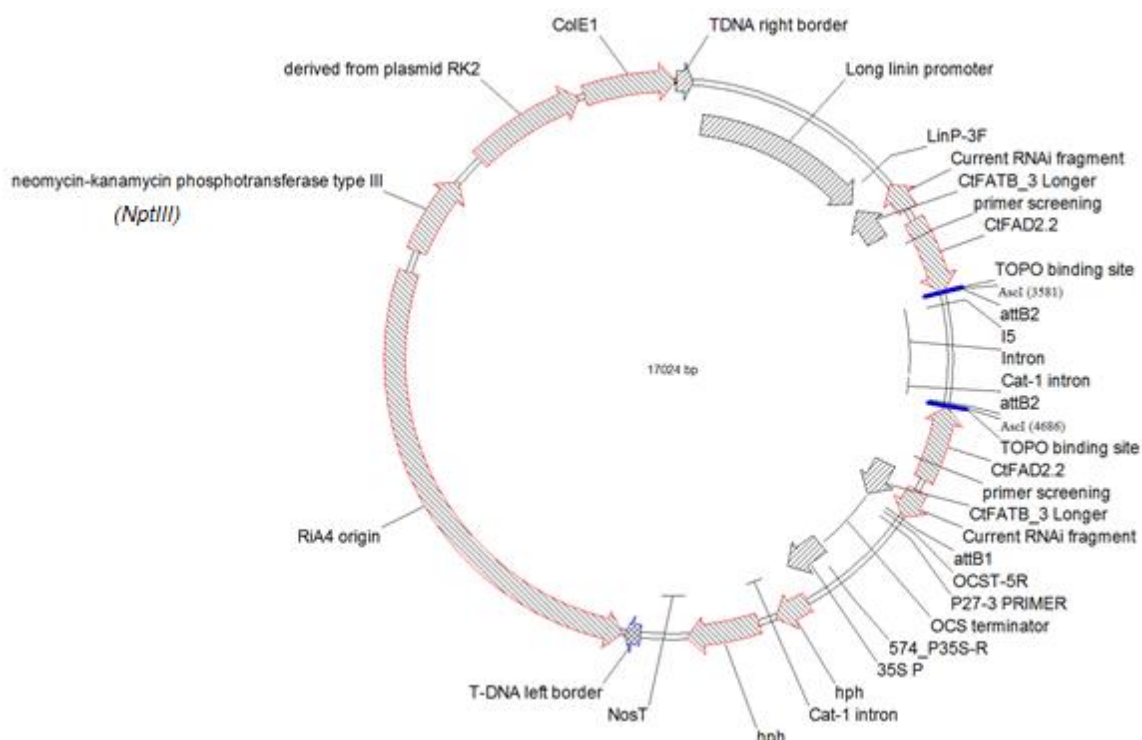


Figure 2: Binary vector pORE-CB1b

3.2 Function and regulation of introduced gene fragments

Information on the genetic elements in the T-DNA used for transformation is summarised in Table 1. The complete pORE-CB1b vector is 17,024 bp comprising 8,053 bp vector backbone, plus 8,647 bp T-DNA (in vector pCW732) and 324 bp of border sequences (163 bp RB and 161 bp.LB). The T-DNA comprises two cassettes (the RNAi cassette and the *hph* cassette). Intervening sequences, where present, have assisted with the cloning of the various components of each cassette.

Table 1: Description of the genetic elements contained in the pCW732 vector of pORE-CB1b

IS = intervening sequence used for DNA cloning

Genetic element	Relative bp location on plasmid	Size - bp/nt	Source	Orient.	Description &Function	Reference
Right Border	6-168	163				
IS	169-303	135				
RNAi cassette						
<i>Linus-lin</i>	304-2295	1992	<i>Linum usitatissimum</i> (flax)	clockwise	<ul style="list-style-type: none"> Promoter region of the linin gene Directs transcription of the <i>CtFATB</i> and <i>CtFAD2-2</i> fragments 	Chaudhary et al.(2010)
IS	2296-2396	100				
<i>CtFATB</i>	2397-2807	412	<i>Carthamus tinctorius</i>	anti-clockwise	<ul style="list-style-type: none"> Fragment of the palmitoyl ACP thioesterase gene 	GenBank: KU059745
IS	2808-2814	7				
<i>CtFAD2-2</i>	2815-3571	757	<i>Carthamus tinctorius</i>	clockwise	<ul style="list-style-type: none"> Fragment of the Δ12 desaturase 	GenBank: KC257448
IS	3572-3660	89				

Genetic element	Relative bp location on plasmid	Size - bp/nt	Source	Orient.	Description & Function	Reference
<i>int1(PDK)</i>	3661-4403	743		forward	<ul style="list-style-type: none"> • Non-coding pyruvate dehydrogenase kinase intron sequence • Part of the spacer sequence between the complementary CtFATB and CtFAD2-2 fragments • Together with <i>int2</i>, aids in stability of the inverted repeat in the bacterial host and increases silencing efficiency 	Wesley et al (2001); Helliwell & Waterhouse (2003)
IS	4404-4444	41				
<i>int2(Cat-1)</i>	4445-4640	196	<i>Ricinus communis</i>	reverse	<ul style="list-style-type: none"> • Non-coding catalase 1 intron sequence • Part of the spacer sequence between the complementary CtFATB and CtFAD2-2 fragments • Together with <i>int1</i>, aids in stability of the inverted repeat in the bacterial host and increases silencing efficiency 	Wang et al (1997); Helliwell & Waterhouse (2003)
IS	4641-4698	57				
<i>CtFAD2-2</i>	4699-5455	757	<i>Carthamus tinctorius</i>	anti-clockwise	<ul style="list-style-type: none"> • Fragment of the $\Delta 12$ desaturase 	GenBank: KC257448
IS	5456-5462	7			•	
<i>CtFATB</i>	5463-5874	412	<i>Carthamus tinctorius</i>	clockwise	<ul style="list-style-type: none"> • Fragment of the palmitoyl ACP thioesterase gene 	GenBank: KU059745
IS	5875-5917	43			•	
<i>ocs</i>	5918-6625	708	<i>Agrobacterium tumefaciens</i>	clockwise	<ul style="list-style-type: none"> • Terminator region of the octopine synthase gene Directs polyadenylation of the CtFATB and CtFAD2-2 fragments 	MacDonald et al (1991)
IS	6626-6672	47				
<i>hph cassette</i>						
35S	6673-7124	452	Cauliflower Mosaic virus (CaMV)	clockwise	<ul style="list-style-type: none"> • Constitutive promoter of the 35S RNA • Directs transcription of the <i>hph</i> gene 	Odell et al(1985); Kay et al. (1987).
<i>hph</i>	7125-7463	339	<i>Escherichia coli</i> via plasmid pVec8-GFP	clockwise	<ul style="list-style-type: none"> • Coding sequence of the hygromycin phosphotransferase gene • Selectable marker • pVec8-GFP = Genbank: ACR26200.1 	Rao et al (1983); Gritz & Davies (1983)
<i>int2(Cat-1)</i>	7464-7653	190	<i>Ricinus communis</i>	forward	<ul style="list-style-type: none"> • In-frame non-coding catalase 1 intron sequence • Presence of an intron in the middle of the gene prevents expression of HPH protein in <i>A. tumefaciens</i> 	Wang et al (1997)
<i>hph</i>	7654-8340	687	<i>Escherichia coli</i> via plasmid pVec8-GFP	clockwise	<ul style="list-style-type: none"> • Coding sequence of the hygromycin phosphotransferase gene • Selectable marker • pVec8-GFP = Genbank: ACR26200.1 	Rao et al (1983); Gritz & Davies (1983)
IS	8341-8365	25			•	
<i>nos</i>	8366-8640	275	<i>Agrobacterium tumefaciens</i>	clockwise	<ul style="list-style-type: none"> • Terminator region of the nopaline synthase gene • Directs polyadenylation of the <i>hph</i> gene 	Bevan et al. (1983); Dhaese et al. (1983)

Genetic element	Relative bp location on plasmid	Size - bp/nt	Source	Orient.	Description & Function	Reference
IS	8641-8816	176				
Left Border	8817-8977	161				

3.2.1 Fatty acid synthesis in plants

Plant oils are primarily composed of triacylglycerols (TAGs) that in turn comprise three fatty acid chains usually 16 or 18 carbons long (Durrett et al. 2008). Synthesis of these fatty acids in plants occurs in plastids and essentially results in the formation of 16:0 (palmitic), 18:0 (stearic) and 18:1 (oleic) fatty acids; odd chained and other even chained fatty acids are produced to a lesser degree.

Acetyl-CoA, the substrate for fatty acid synthesis (Figure 3) is generated from pyruvate and then converted to malonyl-CoA. A series of enzymes then result in the formation of esterified acyl carrier proteins (ACPs) and the generation of two saturated acyl-ACPs (16-carbon palmitoyl-ACP and 18-carbon stearyl-ACP). Fatty acid acyl-ACP thioesterases (FATs) then release them from ACP so that they may be exported to the endoplasmic reticulum for desaturation (addition of double bonds) by fatty acid desaturases (FADs) to produce free 16:0, 18:0 and 18:1 that are assembled into TAGs. There are two classes of FATs – FATA that is predominantly active on oleoyl acyl-ACP substrates, and FATB that uses saturated acyl-ACP substrates (Dörmann et al. 2000).

In relation to the pathway engineered in SHO safflower, two approaches have been taken:

- It has been shown that a higher transcriptional level of FATA relative to FATB leads to a greater production of unsaturated fatty acids (Bonaventure et al. 2003; Huang et al. 2016). In order to favour this ratio the down-regulation of palmitoyl-ACP thioesterase (an FATB class) enzyme that releases palmitic acid from palmitic acid acyl carrier protein (Dörmann et al. 2000) should result in decreased levels of the saturated palmitic acid (Buhr et al. 2002) and increased levels of the monounsaturated oleic acid. The identity of *FATB* genes (there are likely to be more than one) in safflower has yet to be completed.
- The synthesis of polyunsaturated fatty acids in developing oilseeds is normally catalysed by two membrane-associated FADs (Figure 3) that sequentially add a second and third double bond to oleic acid (Kinney 1994). The second double bond, converting oleic acid (18:1) to linoleic acid (18:2), is added at the omega (ω)6 position by a Δ -12 desaturase, encoded by a *FAD2* gene (Okuley et al. 1994; Heppard et al. 1996). The third double bond, converting linoleic acid to linolenic acid (18:3), is added at the ω 3 position by a Δ 15 desaturase, encoded by a *FAD3* gene (Yadav et al. 1993).

In safflower, there are at least 11 *FAD2* genes (Cao et al. 2013), an unusually high number compared to other species. These are non-allelic and are thought to have evolved through gene duplication. While having certain conserved features, the genes show distinct expression patterns and abilities to respond to environmental stimuli. CtFAD2-1 and CtFAD2-2 are the key enzymes controlling the relative accumulation of oleic acid versus linoleic acid in safflower seed oils and a mutation (*oleo*) in the CtFAD2-1 gene which encodes an oleoyl desaturase is responsible for the difference between the high oleic and high linoleic conventional safflower varieties (Liu et al. 2013) i.e. CtFAD2-1 is responsible for the majority of linoleic formation in seeds (Cao et al. 2013) and the mutation causing its down-regulation leads to high oleic acid lines such as M1582, the parent of the SHO lines. It was postulated that CtFAD2-2 is responsible for producing the remainder of the linoleic acid in the high oleic lines and therefore its down-regulation could further increase oleic acid levels.

Thus, it was postulated that down-regulation of both CtFAD2.2 and CtFATB activities in a high oleic safflower background could reduce production of linoleic and palmitic acids and increase the flux of lipids into oleic acid during seed development. This was found to be the case in SHO26 and SHO40 (Wood et al. 2018).

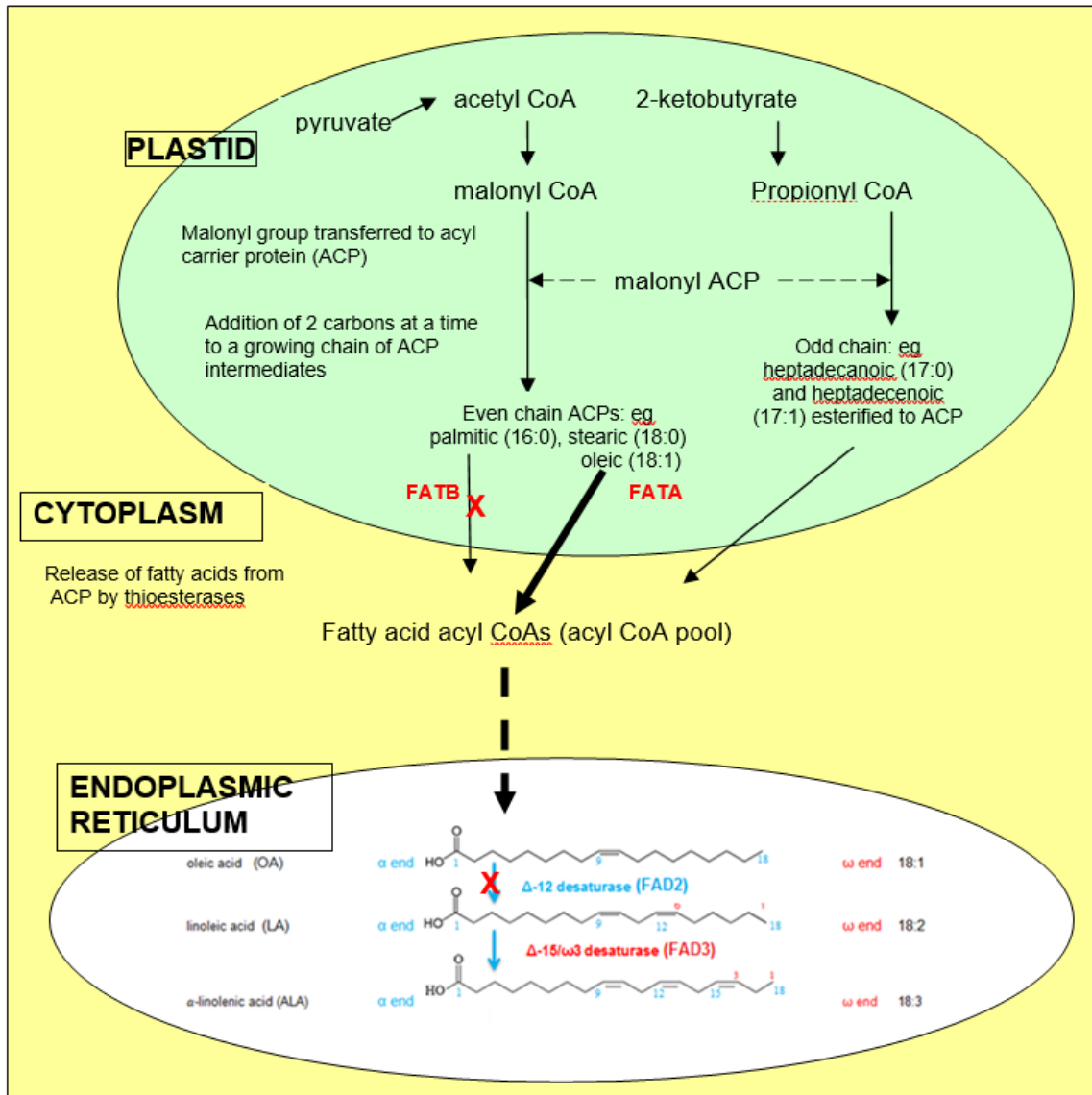


Figure 3: A simplified schematic summary of the synthesis of fatty acids in plants. The sites where the FATB and FAD2 gene products in safflower have been down-regulated is indicated. Diagram adapted from Kinney (1994) and Durrett et al (2008).

3.2.2 The RNAi cassette

The principle of RNAi is that complementary RNA strands, derived from translated inverted repeat sequences, form double-stranded RNA (dsRNA) in a hairpin formation that is recognised by the plant as 'undesirable'. An enzyme known as Dicer is activated and cleaves the dsRNA into short nucleotide sequences (21 – 24 nt). These sequences, in turn, become guides for other enzymes that destroy the complementary messenger RNA (mRNA) sequences produced by translation of the endogenous genes. This effectively silences those genes.

The pCW732 vector was constructed using the pHELLSGATE gene silencing vector system

described by Helliwell & Waterhouse (2003) which facilitates hairpin formation between the inverted repeat sequences contained within pCW732 and thus mediates highly efficient silencing. The inverted repeat sequences are derived from the endogenous *FAD2-2* and *FATB* genes, driven by a single seed-specific promoter (linin) from *L. usitatissimum* (flax) and terminated by the 3' untranslated region of the octopine synthase (*ocs*) gene from *A. tumefaciens*.

The *FAD2-2* gene, used as the target for genetic modification in SHO safflower, is expressed at low levels in the developing embryo and relatively higher levels in cotyledons (Cao et al. 2013). The *FAD2.2* partial sequence introduced into SHO safflower is 757 bp in length. It corresponds to approximately 50% of the open reading frame of the endogenous *FAD2-2* gene.

The *CtFATB* partial sequence introduced into SHO safflower is 412 bp in length and corresponds to approximately 24% of the endogenous gene.

3.2.3 *hph* cassette

The CaMV 35S promoter confers constitutive expression of the *hph* gene that is used as a selectable marker. The *hph* gene (1,139 bp) originates from *E. coli*, W677 carrying the plasmid pJR225 and encodes the enzyme hygromycin B phosphotransferase that catalyses the phosphorylation of hygromycin (Rao et al. 1983). A sequence from the catalase-1 intron, inserted in the middle of the *hph* gene prevents expression of HPH in the *A. tumefaciens* bacterium which, like all bacteria, lacks the ability to splice out intronic sequences to produce a translatable message; this prevents overgrowth of the *Agrobacterium* during the selection process (Wang et al. 1997). The *nos* terminator from the *A. tumefaciens* nopaline synthase gene terminates transcription and provides a polyadenylation site.

3.3 Breeding of SHO safflower

Lines SHO26 and SHO40 underwent slightly different breeding programmes in order to generate regulatory data. These are described in Figure 4. Essentially, T₁ seeds from the T₀ generation were advanced to the T₉ generation through self-fertilisation. At T₄ further breeding strategies were used for the introgression of the SHO safflower event into other safflower lines which in themselves were also used as reference material during field trials for the collection of regulatory data. Sironaria is a high linoleic acid line (see Section 2.1). S317 is a high oleic oil safflower variety that is grown widely in Australia for oil that is used in both the food and industrial/pharmaceutical markets (Hertel 2016). Montola 2003 is a US variety released by the Montana Agricultural Experiment Station in 1999 (Bergman et al, 2005). The variety has not been commercially released in Australia but has undergone extensive field evaluation under genotype code PI612967 (see e.g. Hertel 2016).

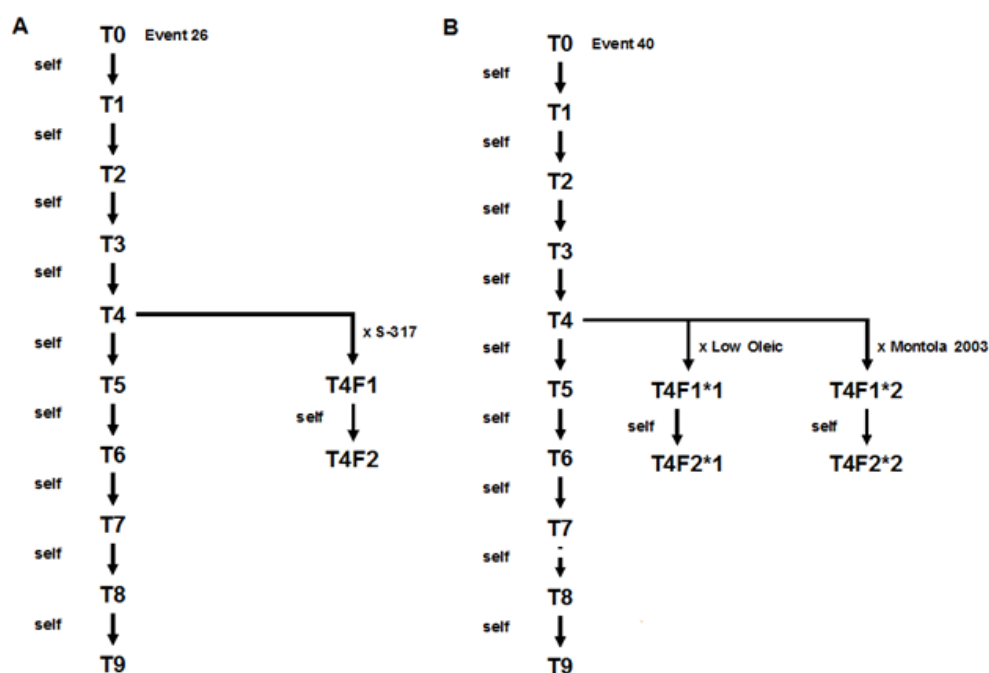


Figure 4: Breeding strategies for SHO26 (A) and SHO40 (B)

The generations and controls used for various analyses described in this report are given in Table 2.

Table 2: SHO safflower generations used for various analyses

Analysis		SHO26 generation(s) used	SHO40 generation(s) used	Control(s) used	Reference material
Molecular characterisation (Section 3.4.)	Insert characterisation	T ₄ T ₆ , T ₇	T ₄ , T ₆ , T ₇	S317	Safflower genome datasets
	Expression of RNAi target genes	T ₄	T ₄	S317	Lesaf496, Centennial, ems/S901
Genetic stability (Section 3.4.2)		T ₄ , T ₇	T ₄ , T ₇	N/A	N/A
Phenotypic stability (Section 3.5.2)	Mendelian inheritance	T ₄ F ₁ , T ₄ F ₂	T ₄ F ₁ * ¹ , T ₄ F ₁ * ² , T ₄ F ₂ * ¹ , T ₄ F ₂ * ²	N/A	N/A
	Fatty acid profile	T ₇	T ₇	M1582	N/A
HPH characterisation (Section 4.1.1)	Western blot	T ₈		M1582	N/A
Compositional analyses (Section 5)		T ₇ , T ₈		M1582	S317, Montola 2003, Sironaria,

3.4 Characterisation of the genetic modification in the plant

A range of analyses were undertaken to characterise the genetic modification in SHO safflower. These analyses focussed on the nature of the insertion of introduced genetic elements and whether any unintended re-arrangements may have occurred as a consequence of the transformation procedure.

3.4.1 Southern Blot Analysis: insert number, insert integrity and plasmid backbone

High quality genomic DNA was isolated and combined from ten plants of each of SHO26 and SHO40 (generation T₄) and also from plants of a non-GM line, S317. Using a published protocol (Belide et al. 2011) the DNA was digested with one of two restriction enzymes (*KpnI* or *PacI*) located within the T-DNA (see Figure 1) but outside the *hph* gene and probed with a radiolabelled P³² probe covering the *hph* gene. This strategy determines the presence of sequences annealing to the hygromycin probe and therefore also provides an estimate of the number of T-DNA insertions in the isolated DNA. Southern blots of both SHO26 and SHO40 DNA showed a single band (indicating a single insertion) while no band was produced in the non-GM line.

3.4.2 Genome Walking: insert number and flanking sequences

A GenomeWalker™ 2.0 Universal Kit ([Clontech](#)²) was used (following the protocols specified) to find unknown genomic DNA sequences adjacent to a known sequence.

High quality genomic DNA was isolated from plants of SHO26 and SHO40 (generations T₄ and T₇), digested with restriction enzymes (*EcoRV* near and upstream of the RB and *DraI* near and downstream of the LB) and used to construct 'libraries' of blunt end DNA fragments that were then ligated to a GenomeWalker adaptor. Each library then underwent PCR amplifications using primer (located at the extremities of the LB or the RB sequence) + adaptor pairs so that sequences flanking the T-DNA were amplified. A single insertion would produce a single amplicon at each of the LB and RB regions.

For SHO26 (plants from both generations), one amplicon of approximately 1,000 bp was produced for regions flanking the LB and one amplicon of approximately 1,400 bp for the RB. For SHO40 (plants from both generations), the LB amplicon was 1,000 bp long and the RB amplicon was 6,000 bp long. These results are consistent with SHO26 and SHO40 containing a single-copy, complete T-DNA insertion with no other partial T-DNA components in the genome and confirm the results of the Southern Blot analysis. The results also indicate the stability of the insert over several generations.

LB and RB amplicons from the genome walking analyses of SHO26 and SHO40 were cloned and sequenced using standard techniques (Sanger sequencing, BigDye® Terminator Chemistry). The sequences were then used as a query sequence into a draft DNA assembly (CSIRO unpublished) of the safflower genome. This draft assembly was based on Illumina-based, next generation short read sequencing datasets on a wild-type safflower genome (commercial supplier), and the read sets were assembled into a draft genome using bioKanga software (CSIRO software; unpublished). The draft assembly covers approximately 80% of the safflower genome in approximately 200,000 fragments/contigs. The flanking sequences for SHO26 were also aligned with a recent genome database of safflower (Bowers et al. 2016). Approximately 1,000 bp of sequence on either side of the insertion site were considered for each of SHO26 and SHO40.

The sequences of the LB and RB amplicons from both SHO26 and SHO40 matched those on the same DNA fragment in the safflower genome database thereby further confirming the presence of a single insertion in each line.

Comparison of SHO26 with the wild type safflower indicated there had been

- a 69 bp deletion within the genomic region
- insertion of the entire (161 bp) LB
- insertion of only 41 bp of the RB
- insertion of 191 bp of non-functional DNA of the replication of origin RiA4 from the

²http://www.clontech.com/US/Products/cDNA_Synthesis_and_Library_Construction/cDNA_and_Genomic_DNA/GenomeWalker_Kits

binary vector pORE-CBIb (see Figure 2) into the junction region immediately downstream of the LB. A potential open reading frame (ORF) was associated with this LB/RiA4 area but no allergenicity/toxicity concerns are raised in the unlikely event a protein were to be expressed (see Section 3.4.5).

Comparison of SHO40 with the wild type safflower indicated there had been

- a 34 bp deletion within the genomic region
- a 37 bp duplication of genomic DNA within the genomic region next to the RB
- insertion of only 14 bp of the LB
- insertion of only 36 bp of the RB

Truncation of border sequences is not uncommon for *Agrobacterium*-mediated transformation events (Tzfira et al. 2004; Kim et al. 2007).

Since the same results for both LB and RB were obtained for both the T₄ and T₇ generations, this is a further confirmation of a stable genome arrangement in SHO26 and SHO40.

3.4.3 Genomic sequences of SHO26 and SHO40

As described above, sequence information from Genome Walking was used to ascertain sequences in the flanking regions of the SHO26 and SHO40 inserts. Sequencing of the full T-DNA insert itself was problematic because of the presence of the inverted repeat in the RNAi cassette. However, PCR was used to confirm the identity of key elements in the insert, particularly in the *hph* cassette and the weight of evidence from the Southern Blot analysis and Genome Walking of the flanking regions suggested that the T-DNA had been inserted intact.

For SHO26, a total of 11,041 bp genomic sequence was obtained. This comprised the full 8,647 bp of the T-DNA (intact and unchanged), 1,042 bp of the 5' border sequence (including 41 bp of RB) and 1,369 bp of the 3' border sequence (including 161 bp of LB and 191 bp of inserted RiA4 DNA from pORE-CBIb).

Similarly, for SHO40, a total of 10,726 bp genomic sequence was obtained. This comprised the full 8,647 bp of the T-DNA (intact and unchanged), 1,098 bp of the 5' border sequence (including 37 bp of duplicated genomic DNA and 36 bp of RB) and 979 bp of the 3' border sequence (including 14 bp of LB).

3.4.4 Plasmid backbone

Genomic DNA was isolated from field-grown plants (generation T₆) of SHO26 and SHO40 and the non-GM line S317 (negative control). Positive controls were plasmid DNA from the binary vector pCW732, and S317 spiked with pCW732. DNA samples were amplified by PCR using five primer pairs covering functional regions of pORE-CBIb outside the T-DNA and including the bacterial origins of replication (RiA4 and ColE1) and the bacterial selection marker *NptIII* that confers resistance to kanamycin (see Figure 5). It is noted that primer P1, covering part of the 3' end of the 4.6 Kb RiA4 origin of replication, did not extend to the terminal 191 bp, identified by sequencing (Section 3.4.2), that had inserted into the junction region in SHO26.

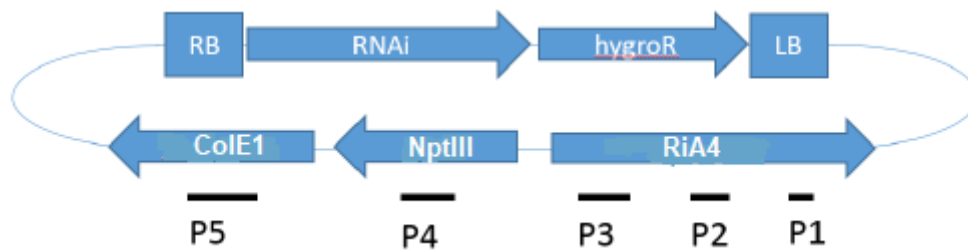


Figure 5: Representation of the backbone sequences covered by the five PCR primers (P1 – P5)

No bands were obtained for the negative control or any samples from SHO26 or SHO40. As expected, the 191 bp from RiA4 was not detected. Single bands were obtained for all positive controls. These results indicated that no functional vector backbone sequences (and hence no antibiotic resistance genes) were present in SHO26 or SHO40.

3.4.5 Open reading frame (ORF) analysis

All open reading frames (ORFs), in the insert itself and the junction regions, created as a result of the pCW732 T-DNA insertion were identified using the ORF detection function within [SnapGene®](#)³ (version 3.3.4). Potential ORFs were defined by start-to-stop (longest continuous predicted amino acid sequence from methionine (ATG) to stop codons (TAA, TAG, or TGA)), in each of the six reading frames where there were at least 30 amino acids. An analysis of potential ORFs in the parent genome in the flanking regions was also done so as to eliminate these from consideration if they occurred in the SHO26 and SHO40 analyses.

While many potential ORFs are likely to be found, few, if any of these are likely to result in an expressed product. ORFs represent only hypothetical coding regions. In order to be translated into a protein, there is an additional requirement for the presence of a promoter, splice sites and/or a terminator (Young et al. 2012). In addition, in eukaryotes, proteins are not usually expressed from the same linear segment of DNA and especially not from tightly packed sequences such as found in the DNA introduced into GM organisms. Since it is necessary for the reading frames to have appropriate ribosomal binding sites in order to be translated into proteins, linear DNA sequences will significantly limit the potential for translation. The most likely sites for ORF expression are in the junction regions since these could be transcribed by regulatory sequences located in the nearby parent genome.

The analysis identified 64 unique ORFs associated with SHO26 (including an ORF incorporating part of the 191 bp RiA4 origin of replication flagged by the sequencing results provided in Section 3.4.2) and 62 unique ORFs associated with SHO40. All of these were analysed using a bioinformatics strategy to determine their similarity to known protein allergens or toxins.

3.4.5.1 Allergenicity database searches

The amino acid sequences identified as ORFs were compared to known allergenic proteins in the Food Allergy Research and Resource Program (FARRP) dataset, which is available through [AllergenOnline](#)⁴ (University of Nebraska). At the date of the search, there were 2,035 sequences in the allergen database (version 17, January 18, 2017). Three types of analyses were performed for this comparison:

- (a) Full length sequence search – a Fast Alignment Search Tool – All (FASTA) alignment was performed comparing the whole of each sequence to the database entries.

³ <http://www.snapgene.com/>

⁴ www.allergenonline.org

Significant homology was determined when there was more than 50% similarity between the query protein and database entry with the E-value threshold set at 10^{-4} . This type of search is considered to be the most predictive of potential allergens (Aalberse 2000).

- (b) 80-mer sliding window search – a FASTA alignment was performed comparing all contiguous 80 amino acids within the ORF to the database entries. Matches were identified if there was greater than 35% homology and an E-value cut-off of 10. This type of search is a precautionary tool to identify smaller regions of high identity between the ORFs and known allergens.
- (c) 8-mer exact match search – A FASTA alignment was performed comparing contiguous 8 amino acids within the ORF to the database entries. Matches were identified if there was 100% homology. It is not known whether an 8-mer of itself is capable of inducing an allergic response (Goodman et al. 2008).

Of the 64 potential ORFs associated with SHO26 and the 62 ORFs associated with SHO40 that were used to query the FARRP database, no similarities were found to any of the known allergenic proteins.

3.4.5.2 Toxin database search

There are no well-curated toxin-specific databases. However, the [UniProt](http://www.uniprot.org)⁵/Swissprot database is an expansive and well-annotated protein database that can be queried for protein homology. Release 2017_05 (10 May 2017) – containing 410,735 entries associated with the word ‘toxin’ - was used to extract proteins containing the keyword, ‘toxin’, in the annotation for protein function. All ORFs were queried against the database with an E-value cut off of 1×10^{-4} .

Of the 64 potential ORFs associated with SHO26 and the 62 ORFs associated with SHO40 that were used to query the UniProt database, no matches were found to any of the known protein toxins.

3.4.6 Analysis of reduced expression of target genes

SHO26 and SHO40 were transformed with a construct containing inverted repeats of gene fragments intended to reduce the expression of two endogenous genes - *CtFAD2-2* and *CtFATB*. Expression of the inverted repeats should create dsRNA which, through natural plant processes, should degrade the mRNA of the two endogenous genes and thus reduce their expression levels. The introduced fragments are driven by a seed-specific promoter i.e. down-regulation should occur only in the seed. A number of approaches were taken to investigate whether this down-regulation had occurred.

3.4.6.1 Lipidomics analysis

A comprehensive lipidomics analysis of seed and non-seed tissues in SHO26 and SHO40 as well as in some representative non-GM safflower lines was done (Wood et al. 2018). The non-GM lines were:

- high oleic S317
- high oleic Lesaf496
- a high oleic line developed by chemical mutagenesis (ems/S901) but that has compromised yield (Weisker 1999)
- low oleic Centennial

Lipids were extracted from various freeze-dried tissue types (cotyledon, hypocotyl, roots, true leaves) of two-week old seedlings and analysed by liquid chromatography-mass

⁵ <http://www.uniprot.org>

spectrometry (LC-MS) based on the method of Reynolds et al (2015).

In roots and true leaves, the profiles of SHO26, SHO40, S317, Lesaf496, and Centennial were very similar and showed the high polyunsaturated fatty acid composition (18:2, 18:3) typical of these vegetative tissues; the profile of *ems/S901* was quite different. In the cotyledon and hypocotyl tissue, the profiles for SHO26, SHO40 and *ems/S901* were very similar and differed from the profiles of the other lines. These results indicated that the RNAi approach used in SHO26 and SHO40 is restricted to seed and developmentally-derived organs such as the emergent cotyledons and hypocotyls.

3.4.6.2 Quantitative PCR of *CtFAD2-2* and *CtFATB* transcripts

Total RNA was extracted from maturing (15 days after pollination) seed of SHO26, SHO40 (T_4 generation) and the non-GM line S317, and then copy DNA (cDNA) synthesis reactions were carried out. Specific primers were then used to amplify the cDNA via quantitative PCR and hence determine the relative mRNA expression levels of each of the endogenous genes following statistical analysis (SPSS Statistics, version23). The results (Figure 6) show that the expression levels of both *CtFATB* and *CtFAD2-2* are significantly reduced in SHO26 and SHO40 compared to the non-GM high oleic acid line S317.

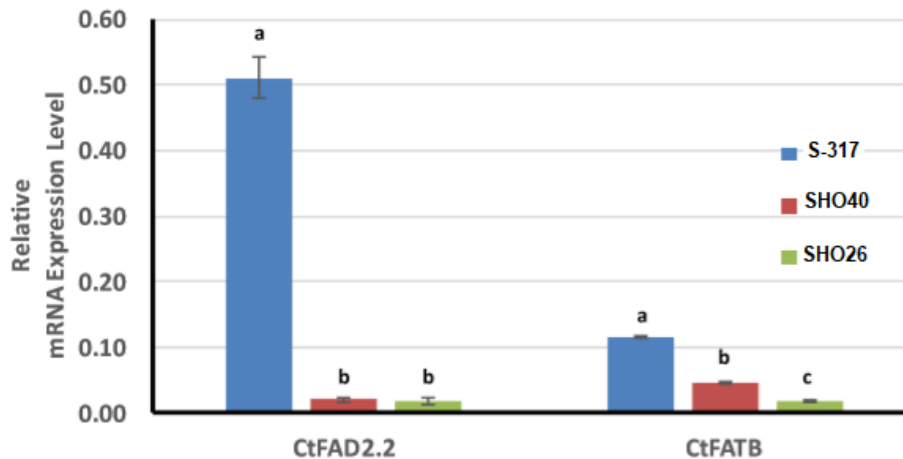


Figure 6: Down regulation of *CtFAD2-2* and *CtFATB* in SHO26 and SHO40
Mean relative mRNA expression levels with the same letter are not significantly different

3.4.6.3 Small RNA analysis

As described in Section 3.2.2, the expression of the RNAi cassette would be expected to result in the production of specific siRNAs having lengths in the range of 21 – 24 nt.

Total RNA was extracted from maturing seed (15 days after pollination) of SHO26, SHO40 (T_4 generation) and a wild type high oleic acid line and then deep sequenced using the Illumina TruSeq small RNA Sample Prep Kit and Illumina based 100 bp single read technologies (John Curtin School of Medical Research, Canberra). Reads of longer than 24 nt were excluded and the resulting siRNA reads were back-aligned to template sequences of *CtFAD2-2* and *CtFATB* obtained from a draft safflower genome (CSIRO) and the T-DNA vector pCW732, using ShortStack (Axtell 2013).

The only siRNAs that were generated in both SHO26 and SHO40 mapped against both *CtFAD2.2* and *CtFATB* precisely within the confines of the hairpin sequence of pCW732 thereby confirming that the silencing observed in SHO26 and SHO40 is indeed mediated by the formation of the expected siRNAs and not by some other component associated with the hairpin structure. The wild type safflower produced no siRNA signal against the templates.

While the wild type contains the *CtFAD2.2* and *CtFATB* genes, these would be transcribed into full length mRNA and no siRNAs would be expected to be produced.

3.5 Stability of the genetic change in SHO safflower

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 event) over successive generations. It is best assessed by molecular techniques. Phenotypic stability refers to the expressed trait remaining unchanged over successive generations. It is often quantified by a trait inheritance analysis to determine Mendelian heritability via assay techniques (chemical, molecular, visual).

3.5.1 Genetic stability

Evidence of genetic stability was provided from the analyses described in Section 3.4.2.

3.5.2 Phenotypic stability

Two approaches were taken to assess phenotypic stability.

3.5.2.1 Mendelian inheritance: seed oil composition

For each of SHO26 and SHO40, an individual plant (T_4 generation) was grown in a glasshouse alongside a non-GM plant with a high oleic genotype (S317 was grown with SHO26 and Montola 2003 was grown with SHO40). Additionally, a plant of SHO40 was also crossed with a plant of a non-GM high linoleic acid genotype (CSIRO safflower breeding line). During flowering, the pairs were manually crossed and all unused florets were emasculated to prevent self-pollination. This procedure generated forced cross seeds – T_4F_1 (Figure 4A) and $T_4F_1^{*1}$ and $T_4F_1^{*2}$ (Figure 4B). The F_1 seedlings were grown to maturity and the flowers were self-pollinated to generate a population of F_2 seed - T_4F_2 (Figure 4A) and $T_4F_2^{*1}$ and $T_4F_2^{*2}$ (Figure 4B). A fatty acid profile (16:0; 18:0; 18:1; 18:2) for the cotyledon tips from imbibed F_2 seeds was then obtained by fatty acid methyl ester analysis (FAME) essentially as described by Zhou et al (2013). The Chi-squared test (χ^2) was used to check whether segregation of the trait (presence = oleic acid > 90%; palmitic acid < 3% for the cross with a high oleic acid line or oleic acid < 90%; linoleic acid >70% for the cross with a high linoleic acid line) fitted the expected 3:1 Mendelian pattern for a single dominant trait.

The results are provided in Table 3 and show, for both lines, that the observed results fitted the expected results and hence that the phenotype was segregating according to Mendelian inheritance.

Table 3: Segregation of the T-DNA insert in SHO26 and SHO40 crosses

Generation/ Background	Total plants	Ratio for 16:0 < 3% presence:absence		X ²	P	Ratio for 18:0 > 90% presence:absence		X ²	P
		Observed	Expected			Observed	Expected		
T ₄ F ₂ (SHO26 x S-137)	119	82:37	80:39	2.36	0.153 (NS)	83:36	80:39	1.75	0.224 (NS)
T ₄ F ₂ * ² (SHO40 x Montola 2003)	59	45:14	44.25:14.75	0.05	0.822 (NS)	45:14	44.25:14.75	0.277	0.599 (NS)
Generation/ Background	Total plants	Ratio for 18:0 < 90% presence:absence		X ²	P	Ratio for 18:1 > 70% presence:absence		X ²	P
		Observed	Expected			Observed	Expected		
T ₄ F ₂ * ¹ (SHO40 x high linoleic)	126	99:27	94.5:31.5	0.68	0.409 (NS)	102:24	94.5:31.5	2.08	0.149 (NS)

3.5.2.2 Fatty acid profile across different environments

The results of this profiling are discussed in the Compositional analysis (Section 5.2.2). The findings were consistent with a stable down-regulation of the endogenous *CtFAD2-2* and *CtFATB* genes in SHO26 and SHO40 across two field trial sites over two years.

3.6 Conclusion

The two SHO safflower lines were generated through *Agrobacterium*-mediated transformation with a single T-DNA containing two expression cassettes. Comprehensive molecular analyses indicate there is one insertion site containing a single, intact copy of the T-DNA. The introduced genetic material is stably inherited across multiple generations and in different genetic backgrounds. No antibiotic resistant marker from the plasmid backbone has been incorporated into the transgenic locus of either line. A small, non-functional sequence of the plasmid backbone from one of the origins of replication has been incorporated into the junction region of SHO26 but does not raise any safety concerns.

Several approaches were taken to analyse the expression of the two genes targeted for silencing. Transcript analysis showed a significant reduction in expression of mRNA from both the *CtFAD2-2* and *CtFATB* genes in SHO26 and SHO40. Lipid analysis indicated down-regulation was confined to tissue developmentally derived from the seed.

The only siRNAs that were generated in both SHO26 and SHO40 mapped against both *CtFAD2.2* and *CtFATB* precisely within the confines of the hairpin sequence of pCW732 thereby confirming that the silencing observed in SHO26 and SHO40 is indeed mediated by the formation of the expected siRNAs and not by some other component associated with the hairpin structure.

4 Characterisation and safety assessment of new substances

The main purpose of the characterisation is to describe the nature of any new substances and their phenotypic and biochemical effects on the organism in which they are expressed, particularly in the parts of the organism consumed as food. Typically, the main focus of the characterisation is on newly expressed (or potentially expressed) proteins, but other (non-protein) substances may need to be considered.

4.1 Newly expressed proteins

In considering the safety of newly expressed proteins it is important to note that a large and diverse range of proteins are ingested as part of the normal human diet without any adverse effects, although a small number have the potential to impair health, e.g., because they are allergens or anti-nutrients (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-nutritional and allergenic effects. To effectively identify any potential hazards requires 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. It is also important to determine if the newly expressed proteins are expressed as expected, including whether any post-translational modifications have occurred.

4.1.1 HPH protein

Unpublished studies submitted:

2018. Protein expression of the hygromycin selectable marker gene in Event 26 and Event 40 leaf tissue and seed. Study report No. GOR-SHOSO-HPH-18-4.0. GO Resources Pty Ltd

Hygromycin B phosphotransferase (HPH) marker protein is an aminocyclitol phosphotransferase that catalyses the phosphorylation of hygromycin and some closely related aminoglycoside antibiotics. Expression of the *hph* gene in plant cells allows for growth and selection of transformed cells in the presence of hygromycin B. HPH encodes a 341 amino acid protein (Figure 7) and has a calculated molecular weight of ca. 42kDa. It catalyses the phosphorylation of the 4-hydroxyl group on the hyosamine moiety of hygromycin B, thereby inactivating it. The enzyme has a narrow range of substrates, in that it phosphorylates hygromycin B, hygromycin B2 and the closely-related antibiotics destomycin A and destomycin B, but does not phosphorylate other aminocyclitol or aminoglycoside antibiotics including neomycin, streptomycin, gentamicin, kanamycin, spectinomycin, tobramycin, and amikacin.

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1 mkkpeltats vekfliiekfd svsdmlqlse geesrafsfd vggrgyvllrv nscadgfykd
61 ryvyrhfasa alpipevldi gefsesltyc isrraaggvtl qdlpetelpa vlqpvaeamd
121 aiaaadlsgt sgfgpfgpqq igqyttwrdf icaiadphvy hwqtvmdtdv sasvaqalde
181 lmlwaedcpe vrhlvhadfg snnvltndgr itavidwsea mfgdsqyeva niffwrpwna
241 cmeqgtryfe rrpelagsp rrraymlrig ldqlyqslvd gnfdadaawaq grcdairvrg
301 agtvgrtqia rrsaavwtdg cvevladsgn rrpstrprak e
```

Figure 7: Predicted amino acid sequence of the HPH protein

The *hph* gene in SHO safflower plants was derived from a plasmid harboured by a hygromycin-resistant isolate of *E. coli*. Hygromycin B phosphotransferases with significant homology to the HPH protein introduced into SHO safflower plants have also been identified in other microbes including *Streptomyces hygroscopicus*, the source of hygromycin B. In plasmid pCW732, the *hph* gene is interrupted by an intronic sequence. This has no effect on the expression of the HPH protein in SHO safflower since the intron is spliced out after transcription.

Hygromycin B is not used in human clinical therapy, but has been used in veterinary medicine (EFSA 2004), principally as an anthelmintic agent in swine and poultry feeds. In animal studies, the protein has no acute toxicity and database analysis reveals no similarity to known toxic proteins or allergens; it is rapidly degraded in gastric fluids (Lu et al. 2007;

Zhuo et al. 2009; OGTR 2017). The US Environmental Protection Agency (EPA) has granted an exemption from the requirement of a tolerance for residues of HPH (EPA 2004).

The HPH protein in SHO safflower was characterised using a weight-of-evidence approach:

- translation of the DNA sequence of the inserts in SHO26 and SHO40 indicate that the expressed HPH protein would have the predicted amino acid sequence (Figure 7).
- the fact the protein was successfully used for selection, indicates the protein has the expected activity.
- expression of the *hph* transcript was estimated following RNA isolation from T₈ generation seeds and leaves of SHO26 and SHO40 and quantitative real time PCR using a number of *hph*-specific primers. The transcript was present in the two SHO lines but not in tissue from controls⁶.
- Western blot analysis of protein from leaf samples of both SHO26 and SHO40 (generation T₈) probed with a primary mouse monoclonal antibody (HYHmb; mybioSource.com product MBS857772; 1:2000 dilution) and a secondary antibody (anti-mouse HRP; 1:5000) showed an expected single band of 40 kDa; there was no evidence of multiple bands. No band was detected in samples from the parental control M1582.

The quantification of HPH protein in seed and leaf tissue was undertaken using an antibody-based method. Total protein was isolated from rosette leaves (true leaf 5 and 6) from 3-week-old plants and from approximately 350 mg seeds (~ 10) and run on sodium dodecyl sulphate polyacrylamide gel electrophoresis followed by immunodetection with hygromycin antibody. Band intensity was quantified using Image Lab™ software. A standard curve was developed using recombinant hygromycin protein from *E. coli* and the safflower samples were compared to this to obtain HPH content through a correlation formula (Limit of Detection – LOD - approx. 0.5 µg). For leaf tissue, values of 130.3 µg/g fresh weight (fw) and 182.3 µg/g fw were obtained for SHO26 and SHO40 respectively. This equated to approximately 0.03% and 0.05% of crude protein. For seeds, values of 168.7 µg/g fw and 39.85 µg/g fw were obtained for SHO26 and SHO40 respectively. This equated to approximately 0.07% and 0.02% of crude protein.

This protein has been previously assessed for safety by FSANZ in Application A509 (FSANZ 2004). The requirement for updated bioinformatics analyses to ascertain whether there are any matches of the protein with known allergens or protein toxins, was incorporated into the database searches done for the ORF analysis of the entire insert (Section 3.4.5). The results support the conclusion that HPH shares no biologically relevant amino acid sequence similarity to either known or putative protein allergens or known or putative protein toxins.

4.2 Newly expressed dsRNA

Evidence has been provided by the applicant to show that the target genes *CtFAD2-2* and *CtFATB* have been suppressed in SHO safflower seed (Section 3.4.6), indicating that the associated dsRNA molecules are being expressed.

It is highly unlikely that the dsRNA molecules formed from the transcription of the DNA sequences introduced into the SHO lines would be translated into a protein that might cause an adverse effect. The hairpin secondary structure that is formed after these fragments are transcribed would prevent engagement of the 40S ribosomal subunit necessary to initiate translation at the 5' end of the RNA (Kozak 1989). The structure of the hairpin would also prevent unwinding of the duplex such that the 40S subunit would be unable to advance along the sequence. Additionally, dsRNA is cleaved into smaller microRNA that would have limited potential for translation. Therefore, it is expected that no novel proteins would be produced

⁶ M1582 (non-GM parent); Sironaria, (Low Oleic type); S317 (High Oleic acid type) – see Section 3.3

as a consequence of these genetic modifications.

In a review by FSANZ (2013), it was concluded the weight of evidence in the published literature on gene silencing does not support the view that dsRNA and RNAi mediators, ingested as part of the normal human diet, have any impact on human gene expression or are likely to have adverse consequences for humans. Nucleic acids, including dsRNA, are already abundantly present in the human diet from both plant and animal sources (Carthew and Sontheimer 2009; Ivashuta et al. 2009). Upon ingestion, enzymes and pH changes in saliva, stomach and intestines degrade nucleic acids into simple components (Hickerson et al. 2008; Martinez et al. 2015; Tittle et al. 2015), which can then be absorbed or excreted. Even if intact or partially degraded nucleic acid molecules arrive in the intestinal region, the large size, hydrophobicity and charged nature of the molecules will limit absorption across the cell barrier lining the intestinal tract. This has been highlighted by the ineffectiveness of gene therapy strategies using naked RNA. Furthermore, there is no scientific basis for suggesting that, when present as a result of the genetic modification of a plant, dsRNA and RNAi mediators possess different properties or pose a greater risk than those already naturally abundant in foods from conventional plants, animals and microorganisms such as yeasts. A history of safe human consumption of RNAi mediators exists, including those with homology to human genes. The evidence published to date also does not indicate that dietary uptake of such RNA from plant food is a widespread phenomenon in vertebrates (including humans) or, if it occurs, that sufficient quantities are taken up to exert a biologically relevant effect.

4.3 Conclusion

SHO26 and SHO40 express one novel protein – HPH. This protein was detectable at low level in leaf and seed tissue. Previous safety assessment of HPH, along with evidence from the literature, indicates the protein would be rapidly degraded in the digestive system following ingestion and would be inactivated by heating. Updated bioinformatic studies considered in this assessment confirm the lack of any significant amino acid sequence similarity to known protein toxins or allergens.

There are no concerns with the safety of the dsRNA or subsequent siRNAs produced in SHO26 and SHO40.

5 Compositional analyses

The main purpose of compositional analyses is to determine if, as a result of the genetic modification, any unexpected changes have 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 analyses 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 (e.g. glycoalkaloids in potatoes).

5.1 Preliminary analysis of oil from SHO26

Super high oleic safflower oil is the primary product intended for use in the industrial oil market. It is also possible the oil may enter the food supply. The seeds may be either cold pressed, expeller pressed or solvent extracted, with the contents of the oil varying on the process (Khalid et al. 2017). The Applicant has advised that oil from SHO safflower, if it entered the food supply, would be refined, bleached and deodorised (RBD) (ISEO 2016), consistent with all other vegetable oils currently used for food purposes. It would therefore be expected that the RBD oil would differ slightly in composition from a cold-pressed oil; in particular, it would be expected that tocopherols and sterols would largely be removed if the oil were deodorised and that the *trans*-fatty acid level would be marginally increased by deodorisation (Ortega-García et al. 2006; NSW DPI 2014; ISEO 2016).

Early on in the development of the SHO lines, and as an indicative comparison (i.e. without statistical analysis), composite samples from four different plots grown at Kununurra (see Figure 8) in 2014 were passed through a seed crush (i.e. cold pressed) and the oil collected. The samples included, in addition to oil from SHO26 (also referred to as 'Event 26') and the non-GM parent M1582, oil from three non-GM varieties covering both the high oleic and low oleic types and four other SHO safflower lines (at this stage, SHO40 had not been selected as a candidate line) all transformed using the same pCW732 vector. As well as confirming whether the genetic modification had the intended effect, the purpose of the analysis was to investigate whether the pCW732 transformation vector itself could lead to any unexpected changes in the properties of SHO safflower oil.

The results (Table 4) showed that, in principle, the down-regulation of CtFATB and CtFAD2-1 resulted, as expected, in a minor decrease in the level of palmitic acid,(16:0), a major decrease in linoleic acid (18:2) and a major increase in oleic (18:1) acid with concomitant changes to the polyunsaturated, monounsaturated and saturated fatty acid levels and to the total omega-6 and omega-9 levels. Since oleic acid is increased, the oxidation stability index (OSI)⁷ is also increased. Other fatty acids remained at levels similar to the control and other high oleic varieties, while differing from the low oleic variety. Tocopherol (with alpha-tocopherol dominant) and sterol levels were fairly similar across all varieties although there have been reports of wide variation in tocopherol levels across safflower generally (see e.g. Matthaus et al. 2015). There was no indication that the genetic modification process resulted in any unexpected changes. A statistically robust analysis of fatty acids in the seed of SHO26 and SHO40 is provided in Section 5.2.2.

⁷ The Rancimat apparatus, used to estimate OSI, accelerates the ageing of an oil by exposing it to heat and air and measuring the time that passes until oxidation takes place (the OSI). The more double bonds a fatty acid has (the more unsaturated it is), the less stable it is i.e. monounsaturated fats have a higher OSI than polyunsaturated fats.

Table 4: Indicative analysis of cold pressed oil produced in a range of safflower lines

Oil Component*	Event 26**	SHOSO**	M1582** High Oleic Type	S-317** High Oleic Type	S901** High Oleic Type	Centennial** Low Oleic Type
Myristic acid C14:0 (%)	0.04	0.04±0.0	0.08±0.01	0.07	0.05	0.11
Palmitic acid C16:0 (%)	2.62	2.51±0.07	4.77±0.12	4.78	3.63	6.87
Palmitoleic acid C16:1 (%)	0.19	0.16±0.0	0.14±0.01	0.15	0.16	0.14
Stearic acid C18:0 (%)	0.48	0.59±0.18	0.60±0.05	0.45	0.59	2.56
Oleic acid C18:1 (%)	91.64	92.25±0.36	76.50±1.12	76.25	89.59	12.84
Linoleic acid C18:2 (%)	1.43	1.19±0.05	14.07±1.04	14.11	2.51	75.31
Linolenic acid C18:3 (%)	0.11	0.08±0.0	0.07±0.02	0.07	0.06	0.08
Arachidic acid C20:0 (%)	0.48	0.44±0.04	0.51±0.01	0.53	0.47	0.36
Eicosenoic acid C20:1 (%)	0.35	0.36±0.02	0.29±0.01	0.30	0.38	0.19
Behenic acid C22:0 (%)	0.31	0.31±0.02	0.35±0.00	0.35	0.39	0.24
Lignoceric acid C24:0 (%)	0.22	0.20±0.01	0.17±0.01	0.18	0.19	0.12
Other Fatty Acids (%)	1.93	1.64±0.13	2.01±0.01	2.30	1.49	0.25
Total Fatty Acids (%)	100	100	100	100	100	100
Polyunsaturated (%)	1.53	1.27±0.04	14.07±1.04	14.18	2.57	75.55
Monounsaturated (%)	92.37	93.02±0.37	77.32±1.12	77.15	90.62	13.95
Saturated (%)	4.16	4.08±0.30	6.49±0.05	6.37	5.32	10.25
Total Omega 3 (mgFA/g)	1.0	0.75±0.05	0.65±0.15	0.70	0.6	2.2
Total Omega 6 (mgFA/g)	13.6	11.20±0.39	134.5±10.2	134.50	23.9	709.6
Total Omega 9 (mgFA/g)	878.3	874.43±6.58	735.65±9.55	731.70	858.9	124.2
Delta Tocopherol (mg/100g)	6.07	4.48±0.48	4.43±0.15	5.05	4.41	3.79
Gamma Tocopherol (mg/100g)	1.01	1.78±0.16	1.71±0.32	1.72	1.37	1.81
Alpha Tocopherol (mg/100g)	57.9	57.68±0.50	56.85±1.45	53.20	59.10	57.50
Campesterol (mg/100g)	59.8	59.68±2.30	49.10±2.30	46.70	51.0	38.70
Stigmasterol (mg/100g)	25.7	21.45±0.80	19.30±1.70	20.90	20.50	18.60
B-sitosterol (mg/100g)	174.0	175.50±7.08	187.0±3.0	161.0	159.0	132.0
Other sterols (mg/100g)	231.0	199.00±7.08	263.50±1.50	256.0	250.0	234.0
OSI (Rancimat) @ 110°C (hours)	38.5	47.55±1.27	13.40±0.60	12.60	32.90	3.22
Viscosity (kinematic @ 40°C; cSt)	39.5	39.15±0.06	37.0±0.20	36.50	38.60	29.2

* Oil samples were analysed by POS Biosciences. Fatty acid levels are presented as a percentage of total fatty acids (%). Where applicable, data are means ± standard error.

** Super-High Oleic Safflower Oil derived from seed samples obtained from field trials conducted by CSIRO under DIR121 at Kununurra in 2014. Oil was extracted from composite samples of 4 independent Safflower events (Event 21, Event 26, Event 30, Event 33). S-317, M1582 and Centennial are conventional safflower. S901 is a conventionally bred high oleic safflower (America Type Culture Collection (ATCC) Number 209181). SHOSO values are means from the independent events Event 21, Event 30, Event 33 and Event 48). M1582 values are the means from 2 separate samples.

5.2 Key components

The OECD publishes [consensus documents](#)⁸, for a number of major crop species, that indicate the key components to be analysed for a comparison between transgenic and conventional lines used for food. Currently, there is no document for safflower. However, a standard range of analyte considerations for food includes proximates, amino acids, fatty acids, minerals, vitamins and anti-nutrients.

The Applicant submitted compositional data for seed, meal (cold press expeller-extracted) and vegetative tissue (from 33 – 38 d old plants, representing succulent plants, from opportunistic germination of seeds left in harvested stubble, that could possibly be used as forage) of SHO safflower. For meal, the analytes measured were proximates and fibre as well as indicators of digestibility and energy. For vegetative tissue the analytes measured were proximate and fibre (plus digestibility/energy indicators), minerals, and tannins and hydrogen cyanide. As meal and forage are considered animal feed, the data were noted by FSANZ but are not reported in this assessment; the results for both types were unremarkable.

Unpublished studies submitted:

2018. Compositional assessment of safflower events GOR-73226-6 and GOR-73240-2. Study report No. GOR-SHOSO-COMP-17-2.2. GO Resources Pty Ltd.

Over the course of field trials conducted for SHO safflower lines, three different sites (Kununurra, Bellata and Kalkee – Figure 8) across major safflower-growing regions of Australia were planted between 2014 and 2017. Trials providing the compositional data reported in this Section were located only in Bellata and Kalkee and were conducted in 2016.



Figure 8: Major locations of SHO safflower field trials

The agronomic practices and pest control measures used were location-specific and were typical for all aspects of safflower cultivation and included soil preparation, fertiliser application, irrigation, and pesticide application. Additionally, a further two or three non-GM cultivars⁹ were also grown as reference lines at each site, in order to generate ranges for each analyte and hence to aid in the determination of the normal variation found in safflower analyte levels. Seed was harvested at physiological maturity – stage 89 (Flemmer et al.

⁸ <http://www.oecd.org/science/biotrack/consensus-document-for-work-on-safety-novel-and-foods-feeds-plants.htm>

⁹ Sironaria, (Low Oleic type); S317 and Montola 2003 (High Oleic types).

2015), with seed moisture levels below 8%. SHO26 and SHO40 samples were verified by PCR analysis for presence of the pCW732 cassettes as well as homozygosity.

For each of SHO26 and SHO40 (generation T₇) and the M1582 control, data were collected from two randomised complete block trials (Kalkee and Bellata) conducted in 2016, each with four replicates. Within each replicate, each safflower variety was planted in plots arranged in random order. At each site, seed was harvested from individual plots and kept separate. Each plot was approximately 1.5 to 2m wide and 10 to 15m in length with a 1m buffer between plots. Replicates of each variety were pooled to form a composite sample.

A point to note is that although normal reproduction in safflower occurs primarily through self-pollination, cross pollination can and does occur. Bees or other insects are generally necessary for optimum fertilisation and maximum yield (Pandey and Kumari 2008). Given the close proximity of the lines at each site it is inevitable that the 'purity' of seed samples may be compromised. To reduce the likelihood of seeds produced by outcrossing being sampled, seeds were not collected from outer rows or ends of plots.

Methods of composition analysis of the seed (kernel + hull) were based on internationally recognised procedures (e.g. those of the Association of Official Analytical Chemists), methods specified by the manufacturer of the equipment used for analysis, or other published methods. All analyses were performed by commercial testing laboratories as follows:

- Proximates, mineral some vitamins, and anti-nutrients: AgriFood Technology, Melbourne
- Remaining vitamins: National Measurements Institute (Laboratories & Trade Measurement), Melbourne
- Amino acids: Australian Proteome Analysis Facility, Sydney
- Fatty acids NSW Department of Primary Industries Oil Testing laboratory, CSIRO, POS Biosciences (Canada)

Descriptive statistics (mean and standard error (SE)) were generated by averaging the results from the composite samples prepared for each site, and are presented in Tables 5 – 10. In order to ensure that the data obtained was not unduly influenced by site, time or other variables, standard errors of the mean (SEM) were examined. The smaller the standard error of the mean, the closer the sample statistic is likely to be to the population parameter.

The compositional components of the two SHO safflower lines and M1582 were statistically analysed using SPSS Statistics (version 2). A one-way Analysis of Variance model was used to establish if there was a variety effect on a particular analyte. If there was significance (a Probability value (P-value) of <0.05), the difference between means was tested using the Least Significant Difference (LSD) test (P-value >0.01). A Levene's Test was performed to verify homogeneity of the variances (p>0.05) and where required, a LOG (base 10) transformation was performed on proportional data to normalise data and obtain homogeneity of the variances.

Any statistically significant differences between SHO safflower and the M1582 control were compared to the 99% tolerance interval compiled from the results for each analyte of the non-GM reference lines combined across sites, to assess whether the differences are likely to be biologically meaningful. These tolerance intervals contain, with 95% confidence, 99% of the levels expressed in the reference populations. Under this set of parameters, some of the lower tolerance intervals calculated were negative numbers; in this instance, the number was rounded up to zero.

Additionally, the results for SHO safflower and M1582 have been compared to a combined

literature range (where available) for each analyte, compiled from published literature for commercially available safflower¹⁰. It is noted, however, that information in the published literature is limited, particularly for high oleic varieties, and that the chemical composition of safflower can be influenced by a variety of genetic, geographical, agronomic and abiotic factors (Salunkhe et al. 1992); even seeds from the head at different positions on the same plant or similar positions on different plants can have variable composition. Therefore, even if means fall outside the published range, this is unlikely to raise a concern.

Seed samples were analysed for proximates, fibre, fatty acids, amino acids, minerals, vitamins, and anti-nutrients (tannins and cyanide). In total, 48 individual analytes (plus energy) and four fatty acid groupings were analysed. Of the individuals, six vitamins had a high number of the observations below the LOD and were excluded from the statistical analysis. The data for 42 individual analytes were therefore considered statistically.

5.2.1 Proximates

The results are given in Table 5 and show there was no significant difference between the means for the SHO lines and those for the control for any of the analytes. In addition, all means fell within both the tolerance interval and the combined literature range.

Table 5: Mean percentage \pm SE of proximates in seed of two SHO safflower lines and the M1528 control collected from two locations

Analyte	M1582	SHO26	SHO40	AOV P-value ⁴	Tolerance interval ²	Combined literature range
Moisture (%)	6.2 \pm 0.17	5.8 \pm 0.17	5.6 \pm 0.10	NS	3.43, 7.53	4.2 – 7.4
Crude Protein (% dw)	21.5 \pm 2.4	20.1 \pm 2.55	21.8 \pm 0.8	NS	4.95, 24.44	14.7 – 37.2
Total Fat (%dw)	31.2 \pm 1.25	34.6 \pm 1.35	34.7 \pm 1.39	NS	11.87, 58.72	13.7 – 29.0
Ash (%dw)	2.9 \pm 0.20	3.1 \pm 0.28	3.19 \pm 0.30	NS	0.5, 6.60	3.4 – 4.2
Carbohydrate ¹ (%dw)	37.7 \pm 4.75	35.85 \pm 3.64	34.2 \pm 2.76	NS	0, 86.38	28.8 – 45.6
Energy (kcalories/100 g) ³	517.52 \pm 4.21	535.2 \pm 5.08	536.3 \pm 4.64	NS	425.9, 663.45	432 - 535

¹ Carbohydrate determined by calculation

² Tolerance interval compiled from analysis of S317, Montola 2003 and Sironaria

³ Calories (Kcal/100 g) = (4 \times % protein) + (9 \times % fat) + (4 \times % carbohydrates)

⁴ NS = Not Significant

¹⁰ Published literature for safflower includes Guggolz et al (1968); Fernandez-Martinez et al. (1993); Rahamatalla et al. (1998); Rahamatalla et al. (2001); Ingale and Shrivastava (2011); Vosoughkia et al. (2011); Yu et al. (2013); Al Surmi et al. (2016), USDA (2018)

5.2.2 Fatty acids

The total oil content of safflower seeds in general ranges from approximately 21% - 40% (Fernandez-Martinez et al. 1993; Matthaus et al. 2015). The fatty acid composition of safflower oils varies with plant species, cultivar and growing conditions, and may also be significantly affected by abiotic stresses (see discussion in Khalid et al. 2017). A very wide tolerance interval would therefore be expected. The major unsaturated fatty acids in safflower are oleic (18:1) and linoleic (18:2). In a survey of 200 safflower accessions from 37 countries, Fernandez-Martinez et al (1993) found that oleic acid levels varied from approximately 3% - 84% and linoleic acid levels varied from approximately 4% - 89% but, in whatever proportion, oleic and linoleic acids make up around 90% of total fatty acids in safflower. The major saturated fatty acids are palmitic and stearic acid which together account for most of the other 10% of total fatty acids.

Fatty acid analyses were undertaken on seed from field trials conducted under OGTR [DIR131](#) in 2016 and 2017. The levels of seven key fatty acids were measured (Table 6). Since most safflower edible oil to date has been derived from high linoleic varieties, fatty acid data on the high oleic acid varieties is limited. In Table 6 the combined literature column reflects the full range (i.e. high oleic and high linoleic) of safflower oil currently available for human consumption. Two tolerance intervals have been calculated, one for both the combined high oleic and high linoleic acid reference varieties and one (in brackets) for just the high oleic acid varieties. Several fatty acid groupings were also analysed.

Table 6: Fatty acid profile (% total fatty acids) in seed of two SHO safflower lines and the M1528 control collected from two locations over two years

Fatty acid	M1582 ¹	SHO26 ¹	SHO40 ¹	AOV P-value	Tolerance interval ²	Combined Literature range ³
Palmitic acid (16:0)	6.27 ± 0.12 ^b	3.40 ± 0.12 ^a	3.34 ± 0.25 ^a	≤0.001	1.13 – 10.47 (2.05 – 8.15)	4 - 12
Palmitoleic acid (16:1)	0.03 ± 0.01 ^a	0.08 ± 0.03 ^a	0.13 ± 0.06 ^a	NS	0.04 – 0.36 (0.13 – 0.27)	0.08 ³
Stearic acid (18:0)	1.76 ± 0.08 ^a	1.40 ± 0.11 ^a	1.71 ± 0.11 ^{ab}	NS	0 – 4.13 (0 – 4.24)	0.7 – 7.6
Oleic acid (18:1)	75.90 ± 0.58 ^b	92.47 ± 0.26 ^a	91.82 ± 0.43 ^a	≤0.001	0 – 100 (62.86 – 88.14)	3 - 84
Linoleic acid (18:2)	15.71 ± 0.58 ^b	2.43 ± 0.20 ^a	2.53 ± 0.26 ^a	≤0.001	0 – 100 (4.94 – 26.46)	4 - 89
Linolenic acid (18:3)	0	0.01 ± 0.0	0	NS	0 – 0.29 (0 – 0.22)	0.15 ⁴
Arachidic acid (20:0)	0.23 ± 0.03 ^a	0.16 ± 0.03 ^a	0.21 ± 0.03 ^a	NS	0.12 – 0.69 (0.05 – 0.75)	Not documented
Saturated	8.27 ± 0.10 ^c	4.96 ± 0.12 ^{ab}	5.26 ± 0.24	≤0.001	0.97 – 15.43 (1.43 – 13.17)	Not documented
Monounsaturated	75.94 ± 0.58 ^b	92.54 ± 0.24 ^a	91.95 ± 0.40 ^a	≤0.001	0 – 100 (63.09 – 88.51)	Not documented
Polyunsaturated	15.71 ± 0.58 ^b	2.43 ± 0.20 ^a	2.60 ± 0.25 ^a	≤0.001	0 – 100 (4.94 – 26.46)	Not documented
<i>trans</i> fatty acids	< 0.2	< 0.2	< 0.2	NS	-	Not documented

¹letters accompanying means indicate results from LSD analysis. Means with the same letter are not significantly different ($p>0.01$). Mauve shading indicates SHO means significantly less than the control; orange shading indicates SHO means significantly higher than the control.

² Unbracketed tolerance interval compiled from S317, Montola 2003 and Sironaria; bracketed tolerance interval compiled from only the high oleic types S317 and Montola 2003

³This combined literature range covers both high oleic and high linoleic acid safflower

⁴indicative value from one cold-pressed oil sample (Orsavova et al. 2015)

The results in Table 6 are as expected and align with those from the preliminary analyses of various SHO lines (see Table 4). As a direct result of the down-regulation of the FAD2-2 and FATB proteins, oleic acid (18:1) levels increased from a mean of 76% (% weight of total fatty acids) in the M1582 control to approximately 92% in SHO26 and SHO40 seeds. In addition, the mean levels of linoleic acid (18:2) decreased from approximately 16% in M1582, to 2.5% in SHO26 and SHO40. The mean levels of palmitic acid were also significantly reduced in the SHO lines compared to the non-transgenic control. As expected, the changes in the mean levels of oleic and linoleic acid in SHO26 and SHO40 were outside the tolerance intervals calculated for both the high oleic reference varieties and the combined varieties. Corresponding to these changes in the individual fatty acids, there were also significant changes in the SHO safflower lines to the overall levels of saturated, monounsaturated and polyunsaturated fatty acids but the total level of unsaturated fatty acids (i.e. monounsaturated + polyunsaturated) was constant at around 91% in the SHO safflower lines and the control. There were no changes in the SHO lines to the level of *trans* fatty acids which was low (< 1%). The presence of small amounts of *trans* fatty acids in most crude vegetable oils is most likely an artefact of the purification process of the crude oil (Tsunami 2012).

5.2.3 Amino acids

Levels of 16 amino acids were measured. Since asparagine and glutamine are converted to aspartate and glutamate respectively during the analysis, levels for aspartate include both aspartate and asparagine, while glutamate levels include both glutamate and glutamine. Results of the analysis are given in Table 7. They show there was no significant difference between the means for the SHO lines and those for the control for any of the analytes. In addition, all means fell within both the tolerance interval and the combined literature range.

Table 7: Mean \pm SE amino acid composition (mg/g dw) in seed of two SHO safflower lines and the M1528 control collected from two locations

Amino acid mg/g dw	M1582	SHO26	SHO40	AOV P- value	Tolerance interval ¹	Combined literature range
Alanine	7.95 \pm 0.98	8.68 \pm 0.08	8.25 \pm 0.40	NS	0, 16.96	0.75 – 26.00
Arginine	16.85 \pm 2.25	18.93 \pm 0.23	17.78 \pm 0.68	NS	0, 37.03	0.37 – 52.80
Aspartate	19.65 \pm 2.3	21.53 \pm 0.14	20.05 \pm 0.73	NS	0, 41.95	0.33 – 32.30
Glutamate	37.68 \pm 4.6	43.15 \pm 0.50	40.03 \pm 1.67	NS	0, 81.66	0.21 – 88.20
Glycine	10.45 \pm 1.24	11.48 \pm 0.17	10.9 \pm 0.40	NS	0, 21.95	60.8 – 11.2
Histidine	5.0 \pm 0.64	5.35 \pm 0.03	5.08 \pm 0.23	NS	0, 10.9	0.24 – 12.3
Isoleucine	7.23 \pm 0.91	7.93 \pm 0.10	7.43 \pm 0.34	NS	0, 15.8	0.11 – 28.10
Leucine	12.3 \pm 1.53	13.65 \pm 0.15	12.8 \pm 0.55	NS	0, 26.51	0.1 – 39.2
Lysine	6.05 \pm 0.72	6.6 \pm 0.04	6.23 \pm 0.29	NS	0, 12.44	0.08 – 46.9
Methionine	0.55 \pm 0.05	1.05 \pm 0.26	1.15 \pm 0.26	NS	0, 1.16	0.09 – 30.01
Phenylalanine	8.4 \pm 1.04	9.23 \pm 0.10	8.75 \pm 0.38	NS	0, 18.25	0.11 – 31.50

Amino acid mg/g dw	M1582	SHO26	SHO40	AOV P-value	Tolerance interval ¹	Combined literature range
Proline	8.53 ± 1.0	9.33 ± 0.13	8.73 ± 0.58	NS	0, 17.12	0.10 – 29.50
Serine	8.33 ± 0.94	9.13 ± 0.16	8.65 ± 0.29	NS	0, 17.15	0.09 – 16.1
Threonine	5.85 ± 0.69	6.5 ± 0.09	6.15 ± 0.22	NS	0, 11.92	0.25 – 10.3
Tyrosine	3.73 ± 0.51	4.23 ± 0.16	4.10 ± 0.10	NS	0, 8.54	0.06 – 23.20
Valine	10.03 ± 1.31	11.03 ± 0.09	10.38 ± 0.53	NS	0, 22.37	0.26 – 24.7

¹ Tolerance interval compiled from Montola 2003 and Sironaria

5.2.4 Minerals

Levels of eight minerals were measured and the means are given in Table 8. There was no significant difference between the means for the SHO lines and those for the control for any of the analytes. In addition, all means fell within both the tolerance interval and the combined literature range.

Table 8: Mean ±SE mineral composition (mg/100 g dw) in seed of two SHO safflower lines and the M1528 control collected from two locations

Mineral mg/100 g dw	M1582	SHO26	SHO40	AOV P-value	Tolerance interval ¹	Combined literature range
Calcium	205 ± 5	215 ± 15	220 ± 20	NS	0, 509.72	59.0 - 1005
Copper	1.4 ± 0	1.3 ± 0.1	1.09 ± 0.31	NS	0, 3.23	0.31 – 0.54
Iron	5.95 ± 0.45	6.25 ± 0.35	5.95 ± 0.65	NS	0, 15.3	3.53 – 157.44
Magnesium	245 ± 5.0	245 ± 5.0	245 ± 15	NS	0, 821.1	30.55 – 607.6
Manganese	1.6 ± 0.2	2.0 ± 0.2	2.2 ± 0.30	NS	0, 4.28	0.26 – 6.28
Phosphorus	550 ± 40	525 ± 35	530 ± 60	NS	0, 2243	33.27 – 770.4
Potassium	650 ± 70	710 ± 50	755 ± 45	NS	0, 1777.2	156 - 1528
Sodium	4.2 ± 2.2	4.3 ± 0.8	4.8 ± 0.2	NS	0, 15.89	12.22 – 134.9

¹ Tolerance interval compiled from Montola 2003 and Sironaria

5.2.5 Vitamins

Levels of ten vitamins were measured. For six of these (Vitamin C, Vitamin B3, Folic Acid, Vitamin B12, Vitamin A and Vitamin D2) the values were considered to be below the LOD. Results for the remaining four vitamins are provided in Table 9. There was no significant difference between the means for SHO26, SHO40 and the control for any of the vitamins. For thiamine and pyridoxine, while the means for SHO26 and SHO40 were higher than the

mean for M1582, there was a wide variation in the levels obtained for each line and this led to a not-significant outcome in the statistical analysis.

Table 9: Mean \pm SE vitamin composition (mg/100 g dw) in seed of two SHO safflower lines and the M1528 control collected from two locations

Vitamin (mg/100 g dw)	M1582e	SHO26	SHO40	AOV P-value	Tolerance interval ¹	Literature value ²
Thiamine (B1)	1.04 \pm 0.36	9.33 \pm 8.67	10.32 \pm 9.86	NS	0, 1.4	1.163
Riboflavin (B2)	0.08 \pm 0.02	0.06 \pm 0	0.08 \pm 0.04	NS	0, 0.39	0.415
Pantothenate (B5)	2.65 \pm 0.35	1.46 \pm 0.95	2.6 \pm 0.3	NS	0, 13.8	4.0
Pyridoxine (B6)	4.68 \pm 4.42	7.1 \pm 0.3	13 \pm 1.0	NS	0, 20.1	1.17

¹ Tolerance interval compiled from S317, Montola 2003 and Sironaria

² Literature value sourced only from USDA National Nutrient Database for Standard Reference (National Agricultural Library 2018) and hence is unlikely to reflect the full range.

5.2.6 Tocopherols and sterols

These analytes were not considered in the detailed compositional analysis but qualitative results for these two categories are provided for SHO26 in Table 4.

Tocopherols and sterols are the major non-saponifiable components of vegetable oils. Safflower seeds are considered to be a rich source of tocopherols. The level of tocopherols (which are anti-oxidants) is governed by the level of unsaturated fatty acids; an increase in unsaturation will result in the formation of higher levels of anti-oxidants to protect the oil. This also applies, to a lesser extent, with some sterols. A slight increase in both would therefore be expected in SHO safflower compared to the parental line and this is borne out in the results (Table 4).

The levels of tocopherols reported in Table 4 across all safflower types including SHO safflower are consistent with those reported in the non-RBT (crude) safflower oil literature where alpha tocopherol predominates at levels of 46.05 – 70.93 mg/100 g and gamma tocopherol is at trace to very low levels (see e.g. Khalid et al. 2017).

Regarding sterols, safflower has been reported to contain 124.8 – 297.6 mg/100 g total sterols with beta-sitosterol being dominant. (see e.g. Vosoughkia et al. 2011). Again the results across all of the safflower lines (including the SHO lines) in Table 4 are consistent with this.

During the deodorisation stage of oil refining both tocopherols and sterols are largely removed i.e. these analytes would only assume nutritional significance if non-deodorised oil, meal or seed were consumed. As mentioned in Section 5.1 it is expected oil for human consumption would be RBD. For reasons provided below (Section 5.2.7) it is unlikely that significant quantities of meal or seed would be consumed in western diets.

5.2.7 Anti-nutrients

Two phenolic glucosides – 2-hydroxy-arctiin and matairesinol monoglucoside – along with the high fibre content of the hulls make safflower meal and seed unsuitable for food (see discussion in e.g. Salunkhe et al. 1992) and hence unlikely to be consumed in western diets.

These analytes are present in the seed and remain in the meal after pressing and contribute to the indigestibility and bitter taste of the meal (Lyon et al. 1979). They are not present in oil, which is the main food product.

Ingale and Shrivastava (2011) identified hydrogen cyanide (prussic acid) and tannins as important anti-nutrients to consider in the use of safflower as a protein source where low quality protein staples may be consumed as food. Tannins are polyphenols that can bind to and precipitate proteins. Hydrogen cyanide can be lethal to most animal species, and plant materials containing ≥ 200 ppm are considered potentially toxic if consumed by humans or livestock (Williams 2012).

The results presented in Table 10 indicate there is no significant difference between the means of SHO26, SHO40 and M1582 for either tannin or hydrogen cyanide.

Table 10: Mean \pm SE anti-nutrient levels in seed of two SHO safflower lines and the M1528 control collected from two locations

Anti-nutrient	M1582	SHO26	SHO40	AOV P-value	Tolerance interval ¹	Combined literature range
Tannin (% dw)	0.13 \pm 0.02	0.13 \pm 0.04	0.18 \pm 0	NS	0 – 2.37	N/A
Hydrogen cyanide (ppm dw)	< 2.5	< 2.5	< 2.5	NS	N/A	N/A

¹For tannin, the tolerance interval was calculated from Montola 2003 and Sironaria; for hydrogen cyanide a tolerance interval could not be calculated as levels were below the level of quantification.

5.3 Conclusions of the compositional analyses

Detailed compositional analyses were conducted on seed from the two SHO safflower lines grown in two different locations in Australia. The seeds were analysed for proximates, amino acids, fatty acids, minerals, vitamins and anti-nutrients. The levels of each analyte in SHO26 and SHO40 were compared to levels in: a) the non-GM parental line, M1582; b) two or three non-GM commercial reference lines grown at the same locations; and c) levels recorded in the literature. A less detailed analysis of SHO26 and other SHO lines during the early selection period of the project was also undertaken at a third location during other growing seasons and included analytes not considered in the detailed analysis.

For the detailed compositional analysis 48 individual analytes (plus energy) and four fatty acid groupings were analysed. Of the individuals, six had a high number of the observations below the LOD and were excluded from the statistical analysis. The data for 42 individual analytes (plus energy) were therefore considered statistically. Of these 42 individual analytes, only three, all fatty acids, differed significantly between SHO26, SHO40 and M1582. The results from the less detailed analysis supported this conclusion. The changes in fatty acid profile were consistent with those expected as a result of the introduction of the RNAi cassette.

The mean fatty acid profile of seed from the SHO lines (Table 6) is expected to be the same for crude oil extracted from the seed. Deodorisation of the oil (as for all RBD oils from all sources) may increase the *trans* fatty acid content but the extent will depend on processing conditions and would not be expected to vary significantly from other retail oils. Deodorisation would also be expected to reduce tocopherols and sterols.

Apart from the intended change to fatty acid profile, seed from SHO26 and SHO40 is otherwise compositionally equivalent to seed from conventional safflower varieties.

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 well-being. In most cases, this can be achieved through an understanding of the genetic modification and its consequences, together with an extensive compositional analysis of the food.

If the compositional analysis indicates biologically significant changes in the levels of certain nutrients in the GM food, additional nutritional assessment may assist to assess 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 (see e.g. OECD 2003; Bartholomaeus et al. 2013; Herman and Ekmay 2014).

6.1 Nutritional considerations of oleic and linoleic acids

Oleic acid, is a non-essential (i.e. it can be synthesised by the liver and therefore is not required in the diet) omega-9 fatty acid that also occurs in a variety of plant and animal sources (e.g. vegetable oils, nuts such as almonds and cashews, beef, chicken and eggs). Linoleic acid is an essential omega-6 fatty acid and must be obtained from food. Good sources include meat, poultry, eggs, walnuts and oils such as soybean and sunflower).

In absolute terms, inclusion of both oleic acid and linoleic acid (and other unsaturated fatty acids) in the diet is considered to offer health and nutritional advantages (see e.g. NHMRC and NZ MoH 2006; Terés et al. 2008, 2008; Huth et al. 2015; Liu et al. 2017; Wang and Hu 2017), particularly in relation to the lowering of LDL-cholesterol and concomitant reduction in the risk of cardiovascular disease, when compared to saturated fats. In terms of both cooking quality and nutrition, the replacement of linoleic acid by oleic acid means that partial hydrogenation is not required to stabilise the fatty acids. This in turn, has the potential to reduce the intake of undesirable *trans* fatty acids in the diet (Huth et al. 2015).

A Mediterranean diet in which olive oil (with high oleic acid content) is an important component (Liu et al. 2017) has been associated with lower levels of plasma lipids and lower blood pressure (Gnoni et al. 2010) that are thought to be mediated by the inhibitory action of oleic acid on the activity of acetyl-CoA carboxylase (that plays an essential role in regulating fatty acid synthesis) and 3-hydroxy-3-methyl-glutaryl CoA reductase (a rate-controlling enzyme in the metabolic pathway that produces cholesterol). However, a number of clinical intervention studies (reported in Huth et al. 2015) have concluded that substitution of high oleic oils for high omega-6 polyunsaturated fat oils did not, in the majority of the trials, have a significant effect on plasma lipids and lipoproteins.

In the case of the two SHO safflower lines, there are significant changes in the fatty acid profile of the seed, specifically an increase in oleic acid and concomitant decrease in linoleic acid compared with the high oleic parent variety, M1582. However, while the levels of oleic and linoleic acids have been altered in SHO26 and SHO40, the total percentage of unsaturated fatty acids (monounsaturated + polyunsaturated) is approximately the same in the M1582 control (91%) and in the two SHO safflower lines (92%) (figures derived from data in Table 6).

A comparison of the indicative level of oleic and linoleic acids in some vegetable oils is provided in Table 11. Of the conventionally bred high oleic oils (arbitrarily designated as

> 70% oleic acid), all, except safflower, are currently available routinely in Australia either for retail sale (mainly olive) or for use by food companies and food service operations (mainly canola and sunflower). All safflower oil (both high oleic and high linoleic) has tended to be more of a 'boutique' product since safflower is not grown extensively in Australia mainly because of the volatility of market prices and the dominance of sunflower, soybean, olive, canola, cottonseed and palm oils for food use. Table 11 shows there are a variety of oils that are available for consumption, with the fatty acid profiles spanning a huge range in both oleic and linoleic acids. While the SHO safflower lines have the highest oleic acid level, the linoleic acid level is not the lowest. It should be noted that no conventionally bred line requires a pre-market safety assessment or estimate of dietary intake impact before entry to the food supply.

Table 11: Levels of oleic and linoleic acids in a range of vegetable oils

Oil source	Oleic acid (18:1) (% total FA) ¹	Linoleic acid (18:2) (% total FA) ²	Reference
Almond*	67.1	25.4	NUTTAB 2010 ¹¹
Avocado*	71	13	(Dreher and Davenport 2013)
Canola*	60.1	20	NUTTAB 2010
Canola (Monola)*	70	20	
Coconut*	6.2	1.6	(Orsavova et al. 2015)
Copha*	1.5	0.3	NUTTAB 2010
Corn*	28.4	56.6	NUTTAB 2010
Cottonseed*	19	54	(ISEO 2016)
Flaxseed*	18	16	Flax Council of Canada (2015)
Grapeseed*	20.7	66.7	NUTTAB 2010
Hemp*	11.5	59.4	(Orsavova et al. 2015)
Macadamia*	65.1	1.3	NUTTAB 2010
Olive*	73.1	8.6	NUTTAB 2010
Palm*	38.3	9.5	NUTTAB 2010
Peanut*	44.4	34.8	NUTTAB 2010
Peanut (SunOleic®)*	80	3	(Gorbet 2003)
Rice bran*	40.7	33.1	NUTTAB 2010
Safflower (high linoleic)*	14	75.6	NUTTAB 2010
Safflower (high oleic)*	78	13	(ISEO 2016)
Safflower - SHO	92	2.5	This application
Sesame*	40.1	42.6	NUTTAB 2010
Soybean*	19.1	57.7	NUTTAB 2010
Soybean (Plenish® high oleic)	76	7	https://www.plenish.com/food/oil-profile/ ¹²
Sunflower*	26.2	62.2	NUTTAB 2010
Sunflower (high oleic)*	82	9	(National Sunflower Association 2018)

¹ yellow highlight = oils with >70% oleic acid

² pink highlight = oils with < 5% linoleic acid

* conventionally bred

¹¹ NUTTAB is an online database, maintained by FSANZ, that contains nutrient data for 2668 foods available in Australia and up to 245 nutrients per food.

<http://www.foodstandards.gov.au/science/monitoringnutrients/nutrientables/nuttab/Pages/default.aspx>

¹² Plenish soybean oil - <https://www.plenish.com/food/oil-profile/>

6.2 Dietary Intake Assessment

Since the prime reason for development of the SHO safflower oils is the super high oleic acid content, a dietary intake assessment was undertaken to estimate dietary intakes of oleic acid both currently and after the introduction of SHO safflower, should the application be approved. There is no established health based guidance value (i.e. an estimated average requirement (EAR)) for oleic acid, since it is non-essential.

The dietary intake assessment used food consumption data from the 2011-12 Australian National Nutrition and Physical Activity Survey (ANNPS), the 2008–09 New Zealand Adult Nutrition Survey and the 2002 New Zealand National Children’s Nutrition Survey. As there were no identified target or at-risk groups, data were analysed for each survey population as a whole. To determine the baseline consumer dietary intake of oleic acid, concentrations of oleic acid in the Australian and New Zealand food supplies were determined primarily using data from Australia’s reference nutrient database (FSANZ in preparation). As oleic acid in the USDA standard reference food composition database (National Agricultural Library 2018) are more comprehensive, these data were used where NUTTAB data were missing or the NUTTAB value was zero. New Zealand food composition data were not used in this assessment. Concentrations of oleic acid in conventional and SHO safflower oils were provided by the applicant.

A number of survey respondents described consuming oil, and its quantity, but could not state what type of oil it was. To account for these ‘unspecified’ oils in the analysis, two different scenarios were developed. In one, the ‘unspecified oil’ was assumed to be vegetable oil, and in the other, all unspecified oil was assumed to be safflower oil. Consumer dietary intakes of oleic acid were estimated for *Baseline (unspecified oils are vegetable oil)*, *Baseline (unspecified oils are safflower oil)* and for two SHO safflower scenarios. The first assumed that SHO safflower oil replaces all safflower oil in conjunction with the Baseline oleic acid dietary intakes (*100% SHO safflower oil scenario*) and the second where SHO safflower oil replaces all safflower oil and all unspecified oil in conjunction with baseline intakes (*100% SHO safflower oil plus 100% SHO unspecified oils scenario*). This second scenario (*100% SHO safflower oil plus 100% SHO unspecified oils scenario*) provides an alternate, higher estimate based on a different assumption which is unlikely to occur because this scenario assumes that all unspecified oils consumed would be SHO safflower oil. In reality SHO safflower oil is mainly intended for industrial use and has a proposed tight specialised CLIP quality assured management program for growing and processing; these two factors will therefore limit the availability of SHO safflower oil for the food supply.

6.2.1 Estimated dietary intakes of oleic acid

6.2.1.1 Australia

The *Baseline (unspecified oils are vegetable oil)* estimated mean and 90th percentile (P90) consumer dietary intakes of oleic acid for Australians aged 2 years and above are 26 g/day and 42 g/day respectively. For the *100% SHO safflower oil scenario*, there are no increases in the estimated mean and P90 consumer dietary intakes of oleic acid from *Baseline (unspecified oils are vegetable oil)* (see Table 12).

For the *100% SHO safflower oil plus 100% SHO unspecified oils scenario* for Australians aged 2 years and above, mean and P90 consumer dietary intakes of oleic acid increase above *Baseline (unspecified oils are safflower oil)* by 3 g/day (8%) to 34 g/day and by 5 g/day (9%) to 56 g/day respectively (see Table 12). It is noted even though this scenario is highly unlikely, the shift in distribution of oleic acid intake would still be within the normal variation of intakes.

6.2.1.2 New Zealand

The *Baseline (unspecified oils are vegetable oil)* estimated mean and P90 consumer dietary intakes of oleic acid for New Zealand children aged 5-14 years are 26 g/day and 43 g/day respectively. Estimated mean and P90 consumer dietary intakes of oleic acid for New Zealand adults aged 15 years of age and above are 29 g/day and 51 g/day respectively. For the *100% SHO safflower oil* scenario there are no increases above the *Baseline (unspecified oils are vegetable oil)* in the estimated mean and P90 consumer dietary intakes of oleic acid for New Zealand children aged 5-14 years and adults aged 15 years and above (see Table 12).

For the *100% SHO safflower oil plus 100% SHO unspecified oils* scenario for New Zealand children aged 5-14 years of age, mean and P90 consumer dietary intakes increase above *Baseline (unspecified oils are safflower oil)* by 4 g/day (13%) to 40 g/day and by 9 g/day (14%) to 70 g/day respectively. For New Zealand adults aged 15 years and above, increases in mean and P90 consumer dietary intake of oleic acid above *Baseline (unspecified oils are safflower oil)* are 4 g/day (11%) to 42 g/day and 8 g/day (12%) to 76 g/day respectively (see Table 12). Although these increases shift the distributions of oleic acid intake for both New Zealand children and adults, the increases are within the normal variation of intakes.

In the *100% SHO safflower oil plus 100% SHO unspecified oils* scenario, a slight population increase in intake of oleic acid was obtained. For reasons already discussed, this scenario would be highly unlikely.

Table 12: Estimated mean and 90th percentile (P90) oleic acid dietary intakes for Australia and New Zealand under two different baseline assumptions and following the replacement of all safflower oil with SHO safflower oil

Country	Age group	Estimated dietary intake of oleic acid (g/day)			
		Scenario: unspecified oils are assumed to be a mix of vegetable oils			
		Mean		P90	
		Baseline	100% SHO safflower oil scenario	Baseline	100% SHO safflower oil scenario
Australia*	2 years and above	26	26	42	42
New Zealand [∇]	5-14 years	26	26	43	43
	15 years and above	29	29	51	51
Country	Age group	Scenario: unspecified oils are assumed to be safflower oil (alternate, higher estimate)			
		Mean		P90	
		Baseline	100% SHO safflower oil plus 100% SHO unspecified oils scenario	Baseline	100% SHO safflower oil plus 100% SHO unspecified oils scenario
Australia*	2 years and above	31	34	51	56
New Zealand [∇]	5-14 years	35	40	62	70
	15 years and above	38	42	67	76

* 2011-12 Australian National Nutrition and Physical Activity Survey (n = 7,735). Based on consumption data from respondents with two days of data only. All respondents were consumers of oleic acid.

[∇] 2002 New Zealand National Children's Nutrition Survey (n = 3,275) and the 2008-09 New Zealand Adult Nutrition Survey (n = 4,721). Based on day 1 consumption data only from all respondents. All respondents were consumers of oleic acid.

6.2.2 Major foods contributing to oleic acid dietary intakes

6.2.2.1 Australia

At *Baseline (unspecified oils are vegetable oil)* for Australians aged 2 years and above, Oils (42%) are the major contributing food category to oleic acid dietary intakes. Within this category, Unspecified oil (25%) and Olive oil (10%) are the main contributors. For Australians, the major contributors to oleic acid to the diet do not change with the replacement of conventional safflower oil with SHO safflower oil in the *100% SHO safflower oil* scenario.

At *Baseline (unspecified oils are safflower oil)* for Australians aged 2 years and above, the major contributor to oleic acid intakes are Oils (52%) (Unspecified oils 37%, Olive oil 9%). When safflower oil was replaced with SHO safflower oil (including unspecified oil) in the *100% SHO safflower oil plus 100% SHO unspecified oils* scenario, the major contributors remained the same with small changes in percentage contribution. Oils contributed 55% (Unspecified oils 42%; Olive oil 8%).

6.2.2.2 New Zealand

At *Baseline (unspecified oils are vegetable oil)* for New Zealand, the major contributor to oleic acid intakes are Oils (53% for children 5-14 years, 47% for adults 15 years and above) (within this category Unspecified oil contributes 44% for children and 36% for adults). In both New Zealand national nutrition surveys, the major contributors to oleic acid in the diet do not change with the replacement of conventional safflower oil with SHO safflower oil in the *100% SHO safflower oil* scenario.

At *Baseline (unspecified oils are safflower oil)* for New Zealand, the major contributor to oleic acid intakes are Oils (65% for children and 59% for adults) (within this category Unspecified oil contributes 59% for children and 51% for adults). In the *100% SHO safflower oil plus 100% SHO unspecified oils* scenario, the major contributor to oleic acid in the diet is similar: Oils (69% for children and 63% for adults) (within this category Unspecified oil 64% for children, 56% for adults).

6.2.3 Conclusion regarding dietary intakes of oleic acid

Oleic acid is non-essential and there are no health based guidance values. As the addition of SHO safflower oil makes little to no difference to oleic acid intakes and food contributors, it is concluded that consumption of SHO safflower will not pose a nutritional concern to the Australian and New Zealand populations.

Further details (including additional data tables) on the dietary intake assessment can be found in a [Supplementary Information](#)¹³ document.

6.2.4 Linoleic acid

Safflower oil can contain a substantial proportion of linoleic acid (see Table 11). However oils and margarines are not the major source of linoleic acid in the Australian diet, even when their use in other products such as salad dressings is included (see Table 13). Consumers ingest fatty acids from a variety of sources, particularly processed foods, with a variety of oleic/linoleic acid levels and are therefore unlikely to rely on a single source for their total fatty acid intake. Australian data from the 2011-12 NNPAS show that the major food

¹³<http://www.foodstandards.gov.au/code/applications/Pages/A1156%e2%80%93FoodderivedfromSuperHighOleicSafflowerLines26and40.aspx>

contributors (>5%) to linoleic acid intake are regular breads and bread rolls, potatoes, nuts and nut products, salad dressings, mixed dishes where poultry or feathered game is the major component and mixed dishes where cereal is the major component (see Table 13) (ABS, 2014). Table 11 indicates there are at least four oils (coconut, copha, macadamia and peanut) available in Australian/NZ supermarkets that contain linoleic acid at a level comparable to that in the SHO safflower oils. Equivalent linoleic acid intake data are not available for New Zealand from national nutrition surveys, but it is likely that the major contributors to linoleic acid intake in New Zealand would be similar to those in Australia.

Table 13: Food contributors to linoleic acid dietary intakes for Australians aged 2 years and above

Major and sub-major food groups	% contribution*
Non-alcoholic beverages	1.0
Cereals and cereal products	15.3
<i>Regular breads, and bread rolls (plain/unfilled/untopped varieties)</i>	7.1
Cereal based products and dishes	21.1
<i>Mixed dishes where cereal is the major ingredient</i>	11.8
Fats and oils	5.7
<i>Margarine and table spreads</i>	4.7
<i>Plant oils</i>	0.4
<i>Unspecified fats</i>	0.1
Fish and seafood products and dishes	5.1
Fruit products and dishes	0.7
Egg products and dishes	2.2
Meat, poultry and game products and dishes	15.3
<i>Mixed dishes where poultry or feathered game is the major component</i>	5.2
Milk products and dishes	2.5
Dairy & meat substitutes	1.4
Soup	1.2
Seed and nut products and dishes	6.5
<i>Nuts and nut products</i>	5.9
Sauces, dips and condiments	7.0
<i>Salad dressings</i>	5.7
Vegetable products and dishes	10.9
<i>Potatoes</i>	5.8
Legume and pulse products and dishes	0.4
Snack foods	2.0
Sugar products and dishes	0.2
Confectionery and cereal/nut/fruit/seed bars	1.5
Alcoholic beverages	0.0
Special dietary foods	0.1
Miscellaneous	0.0
Infant formulae and foods	0.0

Note: All major food groups are shown along with sub-major food groups where the contribution to linoleic dietary intake is ≥5% (as indicated by grey shading)

* % contribution (ABS 2014)

The ABS reported only total polyunsaturated fat intake from the 1995 Australian National Nutrition Survey (NNS) (ABS 1998a). In an analysis of fatty acid intakes from the 1995 NNS data, Meyer et al (2003) commented that the adult (genders combined) median intake of linoleic acid (9.0 g/day) was lower than the mean intake (10.8 g/day) but did not provide any further age or gender breakdowns. Howe et al, (2006) conducted further analysis of the 1995 NNS data and estimated mean and median intakes by type of polyunsaturated fatty acid (including reporting specific mean intakes of linoleic acid). Intakes of linoleic acid based on 1995 NNS data were rounded to form the basis of the Adequate Intake (AI) (NHMRC and NZ MoH, 2006). An AI for linoleic acid was established because there was insufficient data for setting an Estimated Average Requirement and there was no evidence of deficiency in the Australian and New Zealand populations (NHMRC & NZ MoH 2006). The median (P50) and mean usual intakes from the 2011-12 survey are shown in Table 14 and compared to the AIs derived using 1995 NNS data.

Table 14: Usual intakes of linoleic acid for Australians compared to Adequate Intake

Age group (years)	Sex	AI [∇] (g/day)	Usual intake ^ø (P50) (g/day)	Usual intake ^ø (mean) (g/day)
1-3 [¥]	Male	5	5	5
	Female	5	5	5
4-8	Male	8	7	7
	Female	8	6	6
9-13	Male	10	9	9
	Female	8	8	8
14-18	Male	12	10	10
	Female	8	8	9
19 and above	Male	13	8-11	8-11
	Female	8	7-9	7-9

[∇] AI – Adequate intake (NHMRC and NZ MoH 2006). The AIs for the population groups excluding infants were established using intakes from the 1995 NNS.

^ø Usual median and mean intakes of linoleic acid, estimated using 2011-12 Australian National Nutrition and Physical Activity Survey data (ABS 2015).

[¥] AI is for 1-3 years, but the usual intakes are for 2-3 years as this was the age group captured by the nutrition survey.

The intake of linoleic acid has varied over the years. It is noted that the intake of linoleic acid in Western diets over the course of the twentieth century increased dramatically to around 4% of dietary energy (Sanders 2016) concomitant with the shift from using animal fats like butter, lard and tallow to vegetable oils that were naturally high in linoleic acid. This is also evident in trend data for Australia (ABS 2000). With a recent shift to the use of plant cultivars in which linoleic acid has been replaced with the more stable oleic acid, it is likely there has been a decline in linoleic acid intake but current intake is not likely to be lower than it was at the beginning of the twentieth century when no signs of deficiency were noted amongst the general population (Jandacek 2017). In former decades, the ABS produced the Apparent Consumption of Foodstuffs series. The release covering the same year as the 1995 NNS described the trends in consumption of fats and oils related to 1995-96 as “over the longer term, consumption of fats was below the levels recorded in the late 1970s and 1980s, although it was substantially greater than the amount consumed in the 1960s” (ABS 1998b). The consumption of table margarine has risen from 0.4 kg/head in 1938-9 to 5.4 kg/head in 1995-96 and for other margarine from 1.8 kg/head to 2.5 kg/head for the same years, whereas butter consumption decreased from 14.9 kg/head to 2.9 kg per head for the same years (ABS 1998b). Therefore, the AIs, if established at another point in time, would depend on the year of the nutrition survey and could have been higher or lower.

Current intake of linoleic acid is 3.9% of energy averaged across the population (ABS 2014). As noted above, data are insufficient to allow the determination of an Estimated Average Requirement to meet essential fatty acid requirements (NHMRC and NZ MoH 2006) but it

has been estimated to be around 1 – 2% of total dietary energy (Barr et al 1981). Therefore, the AI is likely to overestimate the actual requirements and so the variations seen when comparing the intakes from the 2011-12 NNPAS to the AI would not reflect a safety concern. Given this, and that the dietary intake assessment did not show any increase in oleic acid intakes with replacing all safflower oil with SHO safflower oil in the most likely scenario, and that oils are not the major source of linoleic acid in the diet, it is unlikely that a dietary intake assessment would show a decrease in linoleic acid intake outside of normal daily variation in intakes. Therefore, FSANZ decided not to include linoleic acid in the assessment.

7 References¹⁴

- Aalberse RC (2000) Structural biology of allergens. *Journal of Allergy and Clinical Immunology* 106:228–238. doi: 10.1067/mai.2000.108434
- ABARES, Australian Bureau of Agricultural and Resource Economics and Sciences, Australian Government Department of Agriculture & Water Resources (2017) Agricultural commodities and trade data: Annual commodity statistics 2016. <http://www.agriculture.gov.au/abares/research-topics/agricultural-commodities/agricultural-commodities-trade-data#2016>
- ABS (1998a) 4805.0 – National Nutrition Survey: Nutrient intakes and physical measurements, Australia, 1995. <http://www.abs.gov.au/AUSSTATS/abs@.nsf/DetailsPage/4805.01995?OpenDocument>
- ABS (1998b) 4306.0 Apparent consumption of foodstuffs, Australia, 1994-95 and 1995-96 <http://www.abs.gov.au/AUSSTATS/abs@.nsf/DetailsPage/4306.01994-95%20and%201995-96?OpenDocument>
- ABS (2000) 4306.0 Apparent consumption of foodstuffs, Australia, 1997-98 and 1998-99. <http://www.abs.gov.au/AUSSTATS/abs@.nsf/DetailsPage/4306.01997-98%20and%201998-99?OpenDocument>
- ABS (2014) 4364.0.55.007 – Australian Health Survey: Nutrition first results – foods and nutrients, 2011-12. Australian Bureau of Statistics. <http://www.abs.gov.au/ausstats/abs@.nsf/lookup/4364.0.55.007main+features12011-12>
- ABS (2015) 4364.0.55.008 – Australian Health Survey: Usual nutrient intakes, 2011-12. <http://www.abs.gov.au/AUSSTATS/abs@.nsf/Lookup/4364.0.55.008Main+Features12011-12?OpenDocument>
- Al Surmi NY, El Dengawy R, Khalifa AH (2016) Chemical and nutritional aspects of some safflower seed varieties. *Journal of Food Processing & Technology* 7:5 pp. doi: 10.4172/2157-7110.1000585
- AOF (2015) Section 1: Quality standards, technical information & typical analysis 2015/16. https://www.graintrade.org.au/sites/default/files/file/Commodity%20Standards/2015_2016/201516%20AOF%20Standards%20V14%20-%20August%201%202015.pdf
- Audi J, Belson M, Patel M (2005) Ricin poisoning: a comprehensive review. *JAMA* 294:2342–2351. doi: 10.1001/jama.294.18.2342
- Axtell MJ (2013) ShortStack: comprehensive annotation and quantification of small RNA genes. *RNA* 19:740–751. doi: 10.1261/rna.035279.112
- Baeshen MN, Al-Hejin AM, Bora RS, Ahmed MMM, Ramadan HAI, Saini KS, Baeshen NA, Redwan EM (2015) Production of biopharmaceuticals in *E. coli*: current scenario and future perspectives. *Journal of Microbiology and Biotechnology* 27:953–962. doi: 10.4014/jmb.1412.12079
- Barr LH, Dunn GD, Brennan MF (1981) Essential fatty acid deficiency during total parenteral nutrition. *Annals of Surgery* 193(3): 304 - 311
- Belide S, Hac L, Singh SP, Green AG, Wood CC (2011) Agrobacterium-mediated transformation of safflower and the efficient recovery of transgenic plants via grafting. *Plant Methods* 7:12 pp
- Bevan M, Barnes WM, Chilton M-D (1983) Structure and transposition of the nopaline synthase gene region of T-DNA. *Nucleic Acids Res* 11:369–385
- Bonaventure G, Salas JJ, Pollard MR, Ohlrogg JB (2003) Disruption of the FATB gene in Arabidopsis demonstrates an essential role of saturated fatty acids in plant growth. *The Plant Cell* 15:1020–1033
- Bowers JE, Pearl SA, Burke JM (2016) Genetic mapping of millions of SNPs in safflower (*Carthamus tinctorius* L.) via whole-genome resequencing. *G3* 6:2203–2211. doi: 10.1534/g3.115.026690
- Buhr T, Sato S, Ebrahim F, Xing A, Zhou Y, Mathieson M, Schweiger B, Kinney A, Staswick P, Clements T (2002) Ribozyme termination of RNA transcripts down-regulate seed fatty acid genes

¹⁴ All website references were current as of 30 August 2018

- in transgenic soybean. *The Plant Journal* 30:155–163
- Cao S, Zhou X-R, Wood CC, Green AG, Singh SP, Liu L, Liu Q (2013) A large and functionally diverse family of *Fad2* genes in safflower (*Carthamus tinctorius* L.). *BMC Plant Biology* 13:18 pp
- Carthew RW, Sontheimer EJ (2009) Origins and mechanisms of miRNAs and siRNAs. *Cell* 136:642–655. doi: 10.1016/j.cell.2009.01.035
- Chaudhary S, van Rooijen G, Molony MM, Singh S (2010) Flaxseed specific promoters(US 7,642,346 B2)
- Coutu C, Brandle J, Brown D, Brown K, Miki B, Simmonds J, Hegedus DD (2007) pORE: a modular binary vector series suited for both monocot and dicot plant transformation. *Transgenic Research* 16:771–781. doi: 10.1007/s11248-007-9066-2
- Deblaere R, Reynaerts A, Höfte H, Hernalsteens J-P, Leemans J, van Montagu M (1987) Vectors for cloning in plant cells. *Methods in Enzymology* 153:277–292
- Delaney B, Astwood JD, Cunny H, Eichen Conn R, Herouet-Guicheney C, MacIntosh S, Meyer LS, Privalle LS, Gao Y, Mattsson J, Levine M, ILSI (2008) Evaluation of protein safety in the context of agricultural biotechnology. *Food and Chemical Toxicology* 46:S71-S97
- Dhaese P, Greve H de, Gielen J, Seurinck J, van Montagu M, Schell J (1983) Identification of sequences involved in the polyadenylation of higher plant nuclear transcripts using *Agrobacterium* T-DNA genes as models. *The EMBO Journal* 2:419–426
- Donnenberg MS (2002) *Escherichia coli*: virulence mechanisms of a versatile pathogen. Academic Press, San Diego
- Dörmann P, Voelker TA, Ohlrogge JB (2000) Accumulation of palmitate in Arabidopsis mediated by the acyl-acyl carrier protein thioesterase FATB1. *Plant Physiology* 123:637–643
- Dreher ML, Davenport AJ (2013) Hass avocado composition and potential health effects. *Critical Reviews in Food Science and Nutrition* 53:738–750. doi: 10.1080/10408398.2011.556759
- Durrett TD, Benning C, Ohlrogge J (2008) Plant triacylglycerols as feedstocks for the production of biofuels. *The Plant Journal* 54:593–607
- EFSA (2004) Opinion of the Scientific Panel on Genetically Modified Organisms on the use of antibiotic resistance genes as marker genes in genetically modified plants. *The EFSA Journal* 48:1–18
- EPA (2004) Hygromycin B phosphotransferase; exemption from the requirement of a tolerance. *Federal Register* 69:18275–18278
- FAOSTAT (2017) Online database of the Food and Agriculture Organization of the United Nations. <http://faostat3.fao.org/browse/T/TP/E>
- Fernandez-Martinez J, del Rio M, Haro A de (1993) Survey of safflower (*Carthamus tinctorius* L.) germplasm for variants in fatty acid composition and other seed characters. *Euphytica* 69:115–122
- Flax Council of Canada (2015) Chapter 1: Description and composition of flax. In *Flax – a health and nutrition primer*. pp 9 – 21. https://flaxcouncil.ca/wp-content/uploads/2015/03/FlxPrmr_4ed_Chpt1.pdf
- Flemmer AC, Franchini MC, Lindstrom LI (2015) Description of safflower (*Carthamus tinctorius*) phenological growth stages according to the extended BBCH scale. *Annals of Applied Biology* 166:331–339. doi: 10.1111/aab.12186
- FSANZ (in prep) Australian food composition database, Release 1. Food Standards Australia New Zealand, Canberra, Australia
- FSANZ, Report prepared by Food Standards Australia New Zealand (2004) Application A509 - Food derived from insect-protected cotton line COT102. <http://www.foodstandards.gov.au/code/applications/pages/applicationa509foodd2222.aspx>
- FSANZ, Food Standards Australia New Zealand (2013) Response to Heinemann *et al* on the regulation of GM crops and foods developed using gene silencing. <http://www.foodstandards.gov.au/consumer/gmfood/Pages/Response-to-Heinemann-et-al-on-the-regulation-of-GM-crops-and-foods-developed-using-gene-silencing.aspx>
- Gnoni GV, Natali F, Geele M, Siculella L (2010) Chapter 152 - Oleic acid as an inhibitor of fatty acid and cholesterol synthesis. In: Preedy VR, Watson RR (eds) *Olives and Olive Oil in Health and Disease Prevention*. Elsevier, pp 1365–1373
- Goodman RE, Vieths S, Sampson HA, Hill D, Ebisawa M, Taylor SL, van Ree R (2008) Allergenicity assessment of genetically modified crops - what makes sense? *Nature Biotechnology* 26:73–81
- Gorbet D, University of Florida Extension, Institute of Food and Agricultural Sciences (2003) SunOleic/High Oleic peanuts. <http://ufdcimages.uflib.ufl.edu/IR/00/00/15/85/00001/AG15900.pdf>
- GRDC, Australian Government Grains Research & Development Corporation (2010) Raising the bar with better safflower agronomy: agronomic information and safflower case studies. http://www.australianoilseeds.com/data/assets/pdf_file/0010/6949/GRDC_Raising_the_Bar_-

[Better Safflower Agronomy.pdf](#)

- Gritz L, Davies J (1983) Plasmid-encoded hygromycin B resistance: the sequence of hygromycin B phosphotransferase gene and its expression in *Escherichia coli* and *Saccharomyces cerevisiae*. *Gene* 25:179–188
- Guggolz J, Rubis DD, Herring VV, Palter R, Kohler GO (1968) Composition of several types of safflower seed. *Journal of the American Oil Chemists' Society* 45:689–693. doi: 10.1007/BF02541259
- Halling KC, Halling AC, Murray EE, Ladin BF, Houston LL, Weaver RF (1985) Genomic cloning and characterization of a ricin gene from *Ricinus communis*. *Nucleic Acids Res* 13
- Harrigan E (1987) Voluntary register of oilseeds cultivars in Australia, safflower, cv Sironaria and cv Siothera. *Journal of the Australian Institute of Agricultural Science*:220–221
- Helliwell C, Waterhouse P (2003) Constructs and methods for high-throughput gene silencing in plants. *Methods* 30:289–295
- Heppard EP, Kinney AJ, Stecca KL, Miao G-H (1996) Developmental and growth temperature regulation of two different microsomal ω -6 desaturase genes in soybeans. *Plant Physiol* 110:311–319
- Hertel K (2016) Tactical agronomy of safflower and linseed: place in the rotation, yield potential, time of sowing, plant growth and marketing. <https://grdc.com.au/resources-and-publications/grdc-update-papers/tab-content/grdc-update-papers/2016/03/tactical-agronomy-of-safflower-and-linseed>
- Hickerson RP, Vlassov AV, Wang Q, Leake, D, Ilves, H., Gonzalez-Gonzalez E, Contag CH, Johnston BH, Kaspar RL (2008) Stability study of unmodified siRNA and relevance to clinical use. *Oligonucleotides* 18:345–354. doi: 10.1089/oli.2008.0149
- Horowitz B, Winter G (1957) A new safflower oil with a low iodine value. *Nature* 179:582–583. doi: 10.1038/179582a0
- Howe, P, Meyer B, Record S, Baghurst K (2006) Dietary intake of long chain ω -3 polyunsaturated fatty acids: Contributions of meat sources. *Nutrition* 22:47-53. doi: 10.1016/j.nut.2005.05.009
- Huang J, Zhang T, Zhang Q, Chen M, Wang Z, Zheng B, Xia G, Yang X, Huang C, Huang Y (2016) The mechanism of high contents of oil and oleic acid revealed by transcriptomic and lipidomic analysis during embryogenesis in *Carya cathayensis* Sarg. *BMC Genomics* 17:18 pp. doi: 10.1186/s12864-016-2434-7
- Huth PJ, Fulgoni VL, Larson BT (2015) A systematic review of high-oleic vegetable oil substitutions for other fats and oils on cardiovascular disease risk factors: Implications for novel high-oleic soybean oils. *Adv Nutr* 6:674–693. doi: 10.3945/an.115.008979
- Ingale S, Shrivastava SK (2011) Chemical, nutritional and anti-nutritional study of new varieties of oil seeds from sunflower, safflower and groundnut. *International Journal of Biotechnology Applications* 3:118–129
- ISEO, Institute of Shortening and Edible Oils (2016) Food fats and oils. <http://www.iseo.org/httpdocs/FoodFatsOils2016.pdf>
- Ivashuta SI, Petrick JS, Heisel SE, Zhang Y, Guo L, Reynolds TL, Rice JF, Allen E, Roberts JK (2009) Endogenous small RNAs in grain: Semi-quantification and sequence homology to human and animal genes. *Food and Chemical Toxicology* 47:353–360
- Jandacek RJ (2017) Linoleic acid: a nutritional quandary. *Healthcare (Basel)* 5(2): 25. doi: 10.3390/healthcare5020025
- Kay R, Chan A, Daly M, McPherson J (1987) Duplication of CaMV 35S promoter sequences creates a strong enhancer for plant genes. *Science* 236:1299–1302
- Khalid N, Khan RS, Hussain MI, Farooq M, Ahmad A, Ahmed I (2017) A comprehensive characterisation of safflower oil for its potential applications as a bioactive food ingredient - a review. *Trends in Food Science & Technology* 66:176–186
- Kim S-I, Veena, Gelvin SB (2007) Genome-wide analysis of *Agrobacterium* T-DNA integration sites in the *Arabidopsis* genome generated under non-selective conditions. *The Plant Journal* 51:779–791. doi: 10.1111/j.1365-3113X.2007.03183.x
- Kinney AJ (1994) Genetic modification of the storage lipids of plants. *Current Opinion in Biotechnology* 5:144–151
- Knowles PF, Mutwakil A (1963) Inheritance of low iodine value of safflower selection from India. *Economic Botany* 17:139–145
- Knowles PF, Bill AB, Ruckman JE (1965) High oleic acid content in new safflower, UC-1. *California Agriculture* December 1965:15
- Kozak M (1989) Circumstances and mechanisms of inhibition of translation by secondary structure in eucaryotic mRNAs. *Molecular and Cellular Biology* 9:5134–5142

- Lazo GR, Stein PA, Ludwig RA (1991) A DNA transformation-competent Arabidopsis genomic library in Agrobacterium. *Nature Biotechnology* 9:963–967. doi: 10.1038/nbt1091-963
- Liu Q, Cao S, Zhou X-R, Wood C, Green A, Singh S (2013) Nonsense-mediated mRNA degradation of *CtFAD2-1* and development of a perfect molecular marker for *oleo1* mutation in high oleic safflower (*Carthamus tinctorius* L.). *Theoretical and Applied Genetics* 126:2219–2231. doi: 10.1007/s00122-013-2129-2
- Liu AG, Ford NA, Hu FB, Zelman KM, Mozaffarian D, Kris-Etherton PM (2017) A healthy approach to dietary fats: Understanding the science and taking action to reduce consumer confusion. *Nutr J* 16:53. doi: 10.1186/s12937-017-0271-4
- Lu Y, Xu W, Kang A, Luo, Y. Guo, F., Yang R, Zhang J, Huang K (2007) Prokaryotic expression and allergenicity assessment of hygromycin B phosphotransferase protein derived from genetically modified plants. *Journal of Food Science* 72:M228-M232. doi: 10.1111/j.1750-3841.2007.00437.x
- Lyon CK, Gumbmann MR, Betschart AA, Robbins DJ, Saunders RM (1979) Removal of deleterious glucosides from safflower meal. *Journal of the American Oil Chemists' Society* 56:560–564
- MacDonald HM, Mogen BD, Hunt AG (1991) Characterization of the polyadenylation signal from the T-DNA-encoded octopine synthase gene. *Nucleic Acids Res* 19:5575–5581
- Martinez T, Jimenez AI, Paneda C (2015) Short-interference RNAs: becoming medicines. *Experimental and clinical sciences journal* 14:714–746. doi: 10.17179/excli2015-297
- Matthaus B, Ozcan MM, Al Juhaimi FY (2015) Fatty acid composition and tocopherol profiles of safflower (*Carthamus tinctorius* L.) seed oils. *Natural Product Research* 29:193–196. doi: 10.1080/14786419.2014.971316
- Mündel H-H, Blackshaw RE, Byers JR, Huang HC, Johnson DL, Keon R, Kubik J, McKenzie R, Otto B, Roth B, Stanford K (2004) Safflower production on the Canadian Prairies: Revisited in 2004. <http://publications.gc.ca/site/eng/333269/publication.html>
- Meyer BJ, Mann NJ, Lewis JL, Milligan GC, Sinclair, AJ, Howe PRC (2003) Dietary intakes and food sources of omega-6 and omega-3 polyunsaturated fatty acids. *Lipids* 38(4):391-398
- National Agricultural Library (2018) USDA food composition databases. <https://ndb.nal.usda.gov/ndb/>
- National Sunflower Association (2018) Sunflower oil fatty acid profiles. <https://www.sunflowerusa.com/health/sunflower-oil-fatty-acid-profiles/>
- NHMRC and NZ MoH (2006) Nutrient Reference Values for Australia and New Zealand Including Recommended Dietary Intakes. Australian Government Department of Health and Ageing, National Health & Medical Research Council (Canberra, Australia); New Zealand Ministry of Health (Wellington, NZ)
- NSW DPI, NSW Department of Primary Industries (2014) Variability of quality traits in canola seed, oil and meal - a review. www.dpi.nsw.gov.au/agriculture/broadacre-crops/winter-crops/canola-and-safflower/canola-seeds
- Odell JT, Nagy F, Chua N-H (1985) Identification of DNA sequences required for activity of the cauliflower mosaic virus 35S promoter. *Nature* 313:810–812
- OGTR, Australian Government Office of the Gene Technology regulator (2017) Marker genes in GM plants. <http://www.ogtr.gov.au/internet/ogtr/publishing.nsf/Content/marker-genes-ref-1-htm>
- Oilseeds International Ltd (2016) High Oleic Safflower Oil. <http://www.oilseedssf.com/products/international/ho-safflower-oil.php#fragment-1>
- Okuley J, Lightner J, Feldmann K, Yadav N, Lark E, Browse J (1994) Arabidopsis FAD2 gene encodes the enzyme that is essential for polyunsaturated lipid synthesis. *The Plant Cell* 6:147–158
- Orsavova J, Misurcova L, Ambrozova JV, Vicha R, Mlcek J (2015) Fatty acids composition of vegetable oils and its contribution to dietary energy intake and dependence of cardiovascular mortality on dietary intake of fatty acids. *International Journal of Molecular Sciences* 16:12871–12890. doi: 10.3390/ijms16061287
- Ortega-García J, Gámez-Meza N, Noriega-Rodríguez JA, Dennis-Quíñonez O, García-Galindo HS, Angulo-Guerrero JO, Medina-Juárez LA (2006) Refining of high oleic safflower oil: Effect on the sterols and tocopherols content. *Eur Food Res Technol* 223:775–779. doi: 10.1007/s00217-006-0267-3
- Pandey AK, Kumari A (2008) Pollination ecology of safflower (*Carthamus tinctorius* Linn). In: Knights SE, Potter TD (eds) *Safflower: Unexploited potential and world adaptability: Proceedings of the 7th International Safflower Conference*, Wagga Wagga, New South Wales, Australia
- Popov AM, Kang D (2011) Chapter 118: Analgesic and other medicinal properties of safflower (*Carthamus tinctorius* L.) seeds. In: Preedy VR, Watson RR, Patel VB (eds) *Nuts and seeds in health and disease prevention*. Academic Press, pp 995–1002
- Rahamatalla AB, Babiker EE, Krishna AG, El Tinay AH (1998) Changes in chemical composition, minerals and amino acids during seed growth and development of four safflower cultivars. *Plant*

Foods for Human Nutrition 52:161–170

- Rahamatalla AB, Babiker EE, Krishna AG, El Tinay AH (2001) Changes in fatty acids composition during seed growth and physicochemical characteristics of oil extracted from four safflower cultivars. *Plant Foods for Human Nutrition* 56:385–395
- Rao RN, Allen NE, Hobbs Jr JN, Alborn Jr WE, Kirst HA, Paschal JW (1983) Genetic and enzymatic basis of hygromycin B resistance in *Escherichia coli*. *Antimicrobial Agents and Chemotherapy* 24:689–695
- Reynolds KB, Taylor MC, Zhou X-R, Vanhercke T, Wood CC, Blanchard CL, Singh SP, Petrie J.R. (2015) Metabolic engineering of medium-chain fatty acid biosynthesis in *Nicotiana benthamiana* plant leaf lipids. *Frontiers in Plant Science* 6:164. doi: 10.3389/fpls.2015.00164
- Salunkhe DK, Chavan JK, Adsule RN, Kadam SS (1992) *World oilseeds: chemistry, technology and utilization*. Van Nostrand Reinhold, New York
- Sanders AB (2016) Introduction: The role of fats in human diet. In Sanders AB (ed). *Functional dietary lipids: Food formulation, consumer issues and innovation for health*. Woodhead Publishing Series in Food Science, Technology and Nutrition. pp 1 – 20. <https://doi.org/10.1016/B978-1-78242-247-1.00001-6>
- Smith JR (1996) *Safflower*. AOCS Press, Champaign, Illinois
- Terés S, Barceló-Coblijn G, Benet M, Alvarez R, Bressani R, Halver JE, Escribá PV (2008) Oleic acid content is responsible for the reduction in blood pressure induced by olive oil. *Proceedings of the National Academy of Sciences* 105:13811–13816
- Title AC, Denzler R, Stoffel M (2015) Uptake and function studies of maternal milk-derived microRNAs. *The Journal of Biological Chemistry* 290:23680–23691. doi: 10.1074/jbc.M115.676734
- Tsuzuki W (2012) Study of the formation of trans fatty acids in model oils (triacylglycerols) and edible oils during the heating process. *Japan Agricultural Research Quarterly* 46:215–220
- Tzfira T, Li J, Lacroix B, Cytosky V (2004) *Agrobacterium* T-DNA integration: molecules and models. *Trends in Genetics* 20:375–383. doi: 10.1016/j.tig.2004.06.004
- Ulloa JA, Rosas-Ulloa P, Ulloa-Rangel BE (2011) Physicochemical and functional properties of a protein isolate produced from safflower (*Carthamus tinctorius* L.) meal by ultrafiltration. *Journal of the Science of Food and Agriculture* 91:572–577. doi: 10.1002/jsfa.4227
- Vosoughkia M, Ghavamib M, Gharachorloo M, Sharrifmoghaddasi M, Omidi AH (2011) Lipid composition and oxidative stability of oils in safflower (*Carthamus tinctorius* L.) seed varieties grown in Iran. *Advances in Environmental Biology* 5:897–902
- Wang M-B, Upadhyaya NM, Brettell R, Waterhouse PM (1997) Intron-mediated improvement of a selectable marker gene for plant transformation using *Agrobacterium tumefaciens* 51:325–334
- Wang DD, Hu FB (2017) Dietary Fat and Risk of Cardiovascular Disease: Recent Controversies and Advances. *Annual Review of Nutrition* 37:423–446. doi: 10.1146/annurev-nutr-071816-064614
- Weisker AC (1999) Safflower products with very high levels of unsaturated fatty acids(US 5,912,416)
- Weiss W, Weiland F, Görg A (2009) Protein detection and quantitation technologies for gel-based proteome analysis. *Methods in Molecular Biology* 564:59–82. doi: 10.1007/978-1-60761-157-8_4
- Wesley SV, Helliwell C, Smith NA, Wang M-B, Rouse DT, Liu Q, Gooding PS, Singh SP, Abbott D, Stoutjesdijk PA, Robinson SP, Gleave AP, Green AG, Waterhouse PM (2001) Construct design for efficient, effective and high-throughput gene silencing in plants. *The Plant Journal* 27:581–590
- Williams CJ (2012) *Medicinal plants in Australia*. Volume 3, Plants, potions and poisons. Rosenberg Publishing, Dural, NSW
- Wood CC, Liu Q, Zhou X-R, Green A, Singh SP (2013) High oleic acid oils(WO 2013/159149 A1)
- Wood CC, Okada S, Taylor MC, Menon A, Mathew A, Cullerne D, Stephen SJ, Allen RS, Zhou X-R, Liu Q, Oakeshott JG, Singh SP, Green AG (2018) Seed-specific RNAi in safflower generates a superhigh oleic oil with extended oxidative stability. *Plant Biotechnol J* 2018:1–9. doi: 10.1111/pbi.1291
- Yadav NS, Wierzbicki A, Aegerter M, Caster CS, Pérez-Grau L, Kinney AJ, Hitz WD, Booth Jr JR, Schweiger B, Stecca KL, Allen SM, Blackwell M, Reiter RS, Carlson TJ, Russell SH, Feldmann KA, Pierce J, Browse J (1993) Cloning of higher plant ω -3 fatty acid desaturases. *Plant Physiol* 103:467–476
- Young GJ, Zhang S, Mirsky HP, Cressman RF, Cong B, Ladics GS (2012) Assessment of possible allergenicity of hypothetical ORFs in common food crops using current bioinformatic guidelines and its implications for the safety assessment of GM crops. *Food and Chemical Toxicology* 50:3741–3751. doi: 10.1016/j.fct.2012.07.044
- Yu S-Y, Lee Y-J, Kang S-N, Lee S-K, Jang J-Y, Lee H-K, Lim J-H, Lee OH (2013) Analysis of food components of *Carthamus tinctorius* L. seed and its antimicrobial activity. *Korean Journal of Food*

Preservation 20:227–233

Zayas JF (1997) *Functionality of proteins in food*. Springer

Zhou X-R, Shrestha P, Yin F, Petrie JR, Singh SP (2013) AtDGAT2 is a functional acyl-CoA: diacylglycerol acyltransferase and displays different acyl-CoA substrate preferences than AtDGAT1. *FEBS Letters* 587:2371–2376. doi: 10.1016/j.febslet.2013.06.003

Zhuo Q, Piao JH, Tian Y, Xu J, Yang XG (2009) Large-scale purification and acute toxicity of hygromycin B phosphotransferase. *Biomedical and Environmental Sciences* 22:22–27. doi: 10.1016/S0895-3988(09)60017-9