Application to Food Standards Australia and New Zealand for the Inclusion of Safflower with High Oleic Acid Composition in Standard 1.5.2 Food Produced Using Gene Technology

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Submitted By:

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GO Resources Pty Ltd.

OECD Unique identifiers:
GOR-73226-6 **GOR-7324Ø-2**

***Corresponding Author:**

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Part 1 General Requirements (3.1.1)

A. Executive Summary

GO Resources Pty Ltd (GOR) is submitting this 'Application to FSANZ for the inclusion of safflower with high oleic acid composition in standard 1.5.2 Food Produced Using Gene Technology'.

Safflower seed produces oil that predominantly contain monounsaturated fatty acid (C18:1; oleic acid) and polyunsaturated fatty acid (C18:2; linoleic acid). While both have commercial uses, it is the valuable oleic acid that is used as a replacement to petroleum-based precursors in the manufacture of plastics, lubricants and cosmetics, etc. Traditional breeding programs have developed safflower seed with oleic acid levels in the range of 75–85%, and are the highest purity sources of oleic acid in any oilseed. However, like other oilseeds, the remaining linoleic acid component, at 12-18%, is not desirable for industrial use because it is unstable and difficult to remove during oil processing. Therefore, it is desirable to develop a safflower seed that accumulates high oleic acid (C18:1), but contains very low linoleic acid (C18:2) content.

Two genetically modified (GM) safflower events (OECD Unique IDs: GOR-73226-6 and GOR-7324Ø-2, herein referred to as Event 26 and Event 40 respectively) were developed and molecularly characterised by the Commonwealth Scientific Industrial Research Organisation (CSIRO). The events contain a construct designed to down regulate two safflower fatty acid biosynthesis genes (*CtFATB* and *CtFAD2.2*). Down regulation is achieved using RNAi technology and is targeted to the seed using a promotor from flax (*Linum usitatissimum*). Down regulation of the two safflower genes leads to accumulation of approximately 92% of oleic acid (C18:1) and very low (less than 2%) linoleic acid (C18:2) in the seed, herein referred to as Super High Oleic Acid Safflower Oil (SHOSO). The events also contain a hygromycin resistance gene that was used as a selectable marker during the transformation process. When used as a selectable marker, the hygromycin resistance gene produces a protein that protects plants from Hygromycin B, an aminoglycoside antibiotic.

GO Resources has been working with the CSIRO to characterise the events and collect relevant biosafety information to inform this and other regulatory approval applications. Characterisation has included limited and controlled release field evaluation of GM safflower lines (SHO Safflower) across safflower growing regions of Australia under licences issued by the Office of the Gene Technology Regulator (DIR121 and DIR131). In July 2017, GO Resources submitted an application to the Office of the Gene Technology Regulator for 'dealings involving intentional release (DIR) of genetically modified (GM) plants into the environment - commercial release Commercial Release licence'. The application has been designated the application number DIR158.

SHOSO derived from the safflower containing either Event 26 or alternatively Event 40 is mainly intended for the industrial oil market. However, there may be future sales into the food market therefore GO Resources is also applying to Food Standards Australia and New Zealand (FSANZ) for an amendment to the Food Standards Code (Standard 1.5.2-Food Produced Using Gene Technology). Once all regulatory approvals have been obtained, safflower varieties containing either Event 26 or Event 40 will be marketed for commercial industrial oil production.

GO Resources is committed to the responsible stewardship and management of SHOSO technology. SHOSO technology will be commercialised within a specialised, 'closed-loop' identity preserved (CLIP) quality assured management program. The CLIP program was developed by GO Resources to manage and provide traceability to the production and supply of SHO safflower planting seed to contracted GO Resources SHO safflower grain growers. The CLIP program also provides oversight and traceability of the harvested grain from the grain grower onto grain handlers, seed crushers and processors undertaking SHOSO extraction. Following crushing and processing, the oleic oil will be sold to domestic and export market processors, with the meal being directed to use as a stock feed. (Note: The only product to be exported will be processed oleic oil. That is, no SHO safflower grain will enter the export or domestic grain markets).

Collectively, results of the molecular characterisation, agronomic assessment, and composition analysis support this application for amendment to the *Australia New Zealand Food Standards Code* to allow inclusion of GM safflower events GOR-73226-6 and GOR-7324Ø-2 in **Standard 1.5.2**-*Food Produced Using Gene Technology*.

B. Applicant Details

(a)	Applicant's name/s	
(b)	Company/organisation name	GO Resources Pty Ltd.
(c)	Address (street and postal)	
(d)	Telephone number	
(e)	Email address	
(f)	Nature of the applicant's business	GO Resources Pty Ltd. Oleochemical oil production.
(g)	Details of other individuals, companies or organisations associated with the application	

C. Purpose of the Application

This application seeks to amend the *Australia New Zealand Food Standards Code* to allow for the inclusion of safflower Event 26 and Event 40 in **Standard 1.5.2**-*Food Produced Using Gene Technology*.

In collaboration with the CSIRO, GO Resources has developed and tested safflower events that have super high levels of oleic acid with lower levels of linoleic acid and palmitic acid. The safflower events described in this application have the unique OECD codes: GOR-73226-6 and GOR-7324Ø-2 and are referred to as Event 26 and Event 40 in this submission. GO Resources currently does not intend to export safflower seed to New Zealand or any other jurisdiction. Only extracted oil is intended to be exported. The primary aim of this application is to obtain a food safety approval to protect domestic and international trade. This submission is consistent with GO Resources adherence to the principles and values of the Excellence Through Stewardship® (ETS) program, adhering to stewardship and industry best practice by obtaining regulatory approvals in production and import markets. Further, the application is in support of the Australian Oilseeds Federation best practice document 'Delivering Market Choice for Canola' and the Grain Trade Australia best practice document 'Delivering Market Choice for GM crops'.

D. Justification for the Application

GO Resources has developed new safflower events, Event 26 and Event 40. The new safflower events were created using inserts containing safflower DNA sequences that confer lower levels of two fatty acid biosynthesis genes (*CtFAD2.2* and *CtFATB*), which together contribute to increased accumulation of oleic acid in the seed and lower levels of linoleic acid and palmitic acid.

Super High Oleic Acid Safflower Oil: Sustainable alternatives for raw materials sourced from petroleum-based oils are required. Current alternatives include oleochemical products from the palm, tallow, forestry sources as well as high oleic oilseeds. GO Resources Super High Oleic Oil has a superior oil profile to other oleochemical products. The oil has applications in the lubricant, fine chemical, bioplastics, pharmaceutical and cosmeceutical as well as food and personal care industries.

Costs and Benefits

The SHOSO itself is produced through a simple crushing and filtering process as is used for other oil seeds such as canola. In its marketable form, the oil appears as a light, yellow coloured oil that will be sold in bulk at an expected retail price of $2,500 AUD - $3,500 AUD[1] per tonne.

A high oleic oil with a SHOSO profile does not yet exist in the market. As such, GO Resources has relied on quoted prices for High Oleic that is sold at approximately $2,500 per tonne[2]. Oleic oils derived from algae, at over 90% purity, are said to be available at $1,800 – $3,500 per tonne[3].

The crushing residue (meal) of the pressing to extract the oil, could be a useful additive of mixed stock feeds. GO Resources currently considers this a low priority/low value market.

The key target markets for GO Resources' products are shown in Figure 1. SHOSO has the highest value when used directly in the pharmaceutical and cosmetic markets, where the end products are used on humans and thus have a higher level of concern regarding their safety and therefore require stricter regulations. SHOSO is also a precursor for the manufacture of bioplastic and functional

[1] Sourced from an independent study performed by BM4Tech Limited (UK) on behalf of GO Resources as part of its due diligence on the commercial viability and uses of SHOSO.
[2] Sourced from an Australian company that purchases High Oleic SUN from Asia.
[3] The search for high oleic oils', D. Guzman, Green Chemicals Blog, 2014.

polymers. Additionally, it is a much sought-after ingredient in lubricants and mechanical oils. This market has an annual turnover of $10.5 Billion.

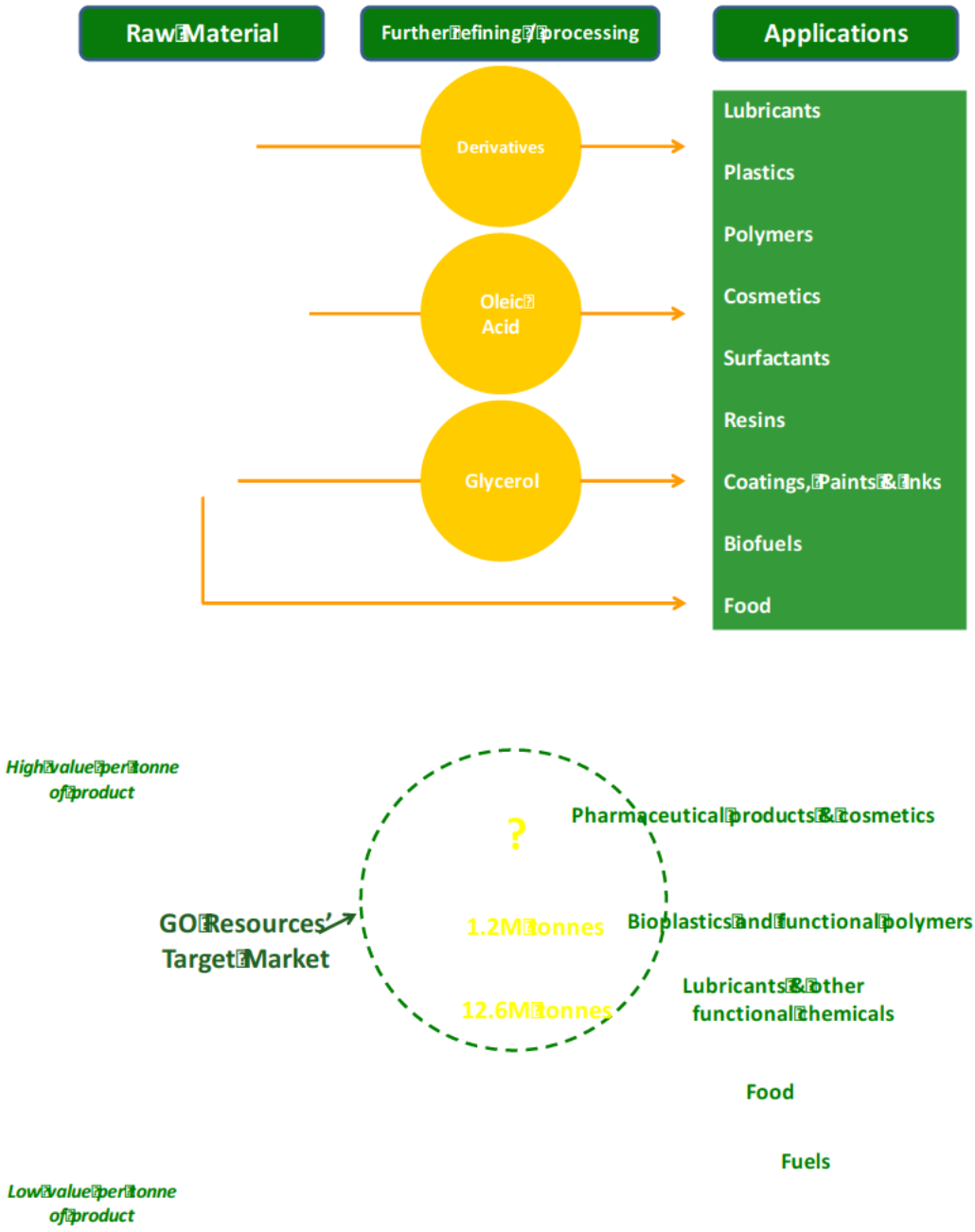

Figure 1. Key target markets for super high oleic safflower oil

E. Information to Support the Application

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

- Part 1: General requirements (3.1.1)
- Part 2: Foods produced using gene technology (3.5.1) main document.

F. Assessment Procedure

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

G. Confidential Commercial Information (CCI)

Confidential Commercial Information (CCI) has been removed from this expurgated copy of the submission document.

Specific information that is considered confidential and removed from this submission copy includes:

- **Figure 8**–The Figure contains sequence information of the flanking sequences associated with the Event 26 insert into the safflower genome. Identity and use of the sequences will form part of a patent filing for Event 26.
- **Figure 10**– The Figure contains sequence information of the flanking sequences associated with the Event 40 insert into the safflower genome. Identity and use of the sequences will form part of a patent filing for Event 40
- **Table 6**–Contains sequence information associated with the flanking sequence of Event 40. The identity and use of the sequences will form part of a patent filing for Event 40
- **Appendix 1**–Contains sequence information of the flanking sequences associated with Event 26 and Event 40 inserts into the safflower genome. Identity and use of the sequences will form part of a patent filing for Event 26 and Event 40.

Release of Information

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

H. Other Confidential Information

No additional confidential material is included in this submission document.

I. Exclusive Capturable Commercial Benefit

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

J. International and Other National Standards

Application for approval of GOR-73226-6 and GOR-7324Ø-2 has been submitted to the Office of the Gene Technology Regulator (DIR158), but has not been submitted to any other jurisdictions.

Responsible environmental stewardship and deployment of biotechnology-derived products are important to GO Resources. Whilst GO Resources is not a member of Excellence Through Stewardship® (ETS), an industry-coordinated initiative that promotes the global adoption of stewardship programs and quality management systems for the full life cycle of biotechnology-derived plant products, it has adopted and does adheres to the principles. The ETS '*Guide for Product Launch Stewardship of Biotechnology-Derived Products*' (ETS, 2013) also references and is consistent with the product launch policies of the Biotechnology Industry Organisation and Crop Life International. Further, this application is consistent with the Australian Oilseeds Federation best practice document 'Delivering Market Choice for Canola' and the Grain Trade Australia best practice document 'Delivering Market Choice for GM crops'.

K. Statutory Declaration – Australia

Statutory Declaration – Australia

Statutory Declarations Act 1959

I, Michael Kleinig, CEO/Managing Director for GO Resources Pty Ltd, 15 Sutherland Street Brunswick Victoria Australia 3056, make the following declaration under the *Statutory Declarations Act 1959*:

1. the information provided in this application fully sets out the matters required
2. the information provided in this application is true to the best of my knowledge and belief
3. no information has been withheld that might prejudice this application, to the best of my knowledge and belief

I understand that a person who intentionally makes a false statement in a statutory declaration is guilty of an offence under section 11 of the *Statutory Declarations Act 1959*, and I believe that the statements in this declaration are true in every particular.

Before me,

L. Checklists Provided With Application

General Requirements

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

Foods Produces Using Gene Technology

Foods produced using gene technology (3.5.1)		
Check	**Page No.**	**Mandatory requirements**
☒	18	A.1 Nature and identity
☒	19	A.2 History of use of host and donor organisms
☒	21	A.3 Nature of genetic modification
☒	55	B.1 Characterisation and safety assessment
☒	59	B.2 New proteins
☒	59	B.3 Other (non-protein) new substances
☐	n/a	B.4 Novel herbicide metabolites in GM herbicide-tolerant plants
☒	68	B.5 Compositional analyses
☒	85	C Nutritional impact of GM food
☒	85	D Other information

Part 2 Specific Data Requirements for Safety Assessment

The following information is provided to support an application for a new genetically modified food. The details presented are in accordance with Section 3.5.1. of the FSANZ Application Handbook as at 1 March 2016.

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Abbreviations, Acronyms and Definitions[4]

Abbreviation	Definition
ADP	Adenosine diphosphate
A. tumefaciens	*Agrobacterium tumefaciens*
ATP	Adenosine triphosphate
AUG	Start codon
Backbone DNA	DNA associated with construct backbone
bp	Base pair
DNA insert	DNA sequence from pCW732 integrated into the safflower genome
dNTP	Deoxy nucleotide triphosphate
dsRNA	Double-stranded RNA
ETS	Excellence Through Stewardship
FARRP	Food Allergy Resource Research Program and the University of Nebraska Lincoln
FDA	Food and Drug Administration
GOR-73226-6	Event 26
GOR-73240-2	Event 40
ILSI	International Life Sciences Institute
kb	Kilobase
LB	Left border
LOD	Limit of detection
LOQ	Limit of quantification
nt	Nucleotide(s)
NGS	Next generation sequencing
NTO	Non-target organism
OECD	Organisation for Economic Cooperation and Development
ORF	Open reading frame
PCR	Polymerase chain reaction
qPCR	Quantitative PCR
RB	Right border
RT-qPCR	Reverse transcription-qualitative polymerase chain reaction
SHOSO	Super high oleic safflower oil
WT	Wild-type

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

A. Technical Information on the Food Produced Using Gene Technology

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

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

An oleic type safflower advanced breeding line obtained from Mexico (M1582) was transformed with *Agrobacterium tumefaciens* containing a binary vector with a plasmid pCW732 (according to Belide et al., 2011) to generate the SHOSO Event 26 and Event 40. The events were developed to confer an increased level of oleic acid in the seed as well as a reduction in the levels of linoleic acid and palmitic acid (Table 1).

The modifications included:

1. Down regulation of the endogenous *Carthamus tinctorius* L palmitoyl-ACP thioesterase (*CtFATB*) and *Carthamus tinctorius* L. Δ12 desaturase (*CtFAD2.2*) fatty acid biosynthesis genes in safflower seed leading to increased oleic acid (C18:1) levels and decreased linoleic acid (C18:2) levels.
2. Expression of the hygromycin phosphotransferase gene (*hph*) used as a selectable marker during the transformation process.

Table 1: Summary of genes, intended traits, and benefits in Event 26 and Event 40

Construct	Gene Target	Mechanism	Intended Trait	Intended Benefit
pCW732	*CtFAD2.2*	RNAi down regulation	Reduces Δ12 desaturase	Contributes to increased oleic acid, decreased linoleic and palmitic acids in the seed
	CtFATB	RNAi down regulation	Reduces palmitoyl-ACP thioesterase	Contributes to increased oleic acid, decreased linoleic and palmitic acids in the seed
	HPH	Over expression	Antibiotic resistance	Selection *in vitro* only

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

In accordance with OECD '*Guidance for the Designation of a Unique Identifier for Transgenic Plants*', the OECD Unique Identification Code for the safflower events are: GOR-73226-6 and GOR-7324Ø-2.

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

Marketing names for the safflower containing the SHOSO technology in the oleochemical market have yet to be determined. However, the safflower oil will be marketed under a variety of labels as super high oleic safflower oil, depending on the licenced user of the event.

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

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

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

Donor DNA in the insert consists of both coding and non-coding genetic elements from the plasmid pCW732 as described in Table 2. Details of the pathogenicity, toxicity or allergenicity of safflower are described in the OGTR biology document 'The Biology of *Carthamus tinctorius* L. (safflower)' (OGTR, 2015). Safflower are not known to cause disease in humans or animals and have a long history of safe use as a bird food.

Increased oleic acid content has also been assessed by FSANZ in soybean (see FSANZ Applications A387 and A1018).

The hygromycin resistance gene is commonly used in gene technology and there is no evidence that the HPH protein is either toxic or allergenic (OGTR 2012). A safety assessment of the hygromycin resistance gene in cotton has previously been undertaken by FSANZ (see A509 and A615).

Bioinformatic analysis of the non-coding elements and ORF analysis of Event 26 and Event 40 in safflower did not indicate homology to pathogenic, toxic or allergenic compounds (see Section B).

Table 2. Genetic elements from pCW732 introduced into events GOR-73226-6 and GOR-7324Ø-2

Genetic Element	Function in the GM plant	Source Organism	Gene Accession Number	Reference
Flax linin	Flax linin promoter (2032bp)	*Linum usitatissimum*	Patent US 7,642,346	–
CtFATB	*Carthamus tinctorius* L palmitoyl-ACP thioesterase (300bp)	*Carthamus tinctorius L.*	–	–
CtFAD2.2	*Carthamus tinctorius* L. Δ12 desaturase (754bp)	*Carthamus tinctorius L.*	–	–
int1	non-coding Pyruvate Dehydrogenase Kinase (PDK) intron sequence (742bp)	–	–	(Wesley et al 2001; Helliwell and Waterhouse 2005)
int2	non-coding Catalase1 sequence from castor bean; intron sequence (196bp)	*Ricinus communis*	–	(Helliwell and Waterhouse 2005)
ocs	octopine synthase polyA (743bp); terminator and polyadenylation signal	*Agrobacterium tumefaciens*	–	(MacDonald et al., 1991)
35S	Promoter with duplicated enhancer region (462bp)	Cauliflower mosaic virus	–	(Odell et al., 1985; Kay et al., 1987; Sanders et al. 1987)
hph	Hygromycin phosphotransferase antibiotic resistance gene and selectable marker (1237bp)	*Streptomyces sp.*	–	(Gritz and Davies, 1983; Bilang et al., 1991; Murray et al., 2004)
nos 3'	3' non-translated region of the nopaline synthase gene (239bp); terminator and polyadenylation signal	*Agrobacterium tumefaciens*	–	(Bevan et al., 1983; Rogers et al, 1986).
pORE-CBIb	Plant binary expression vector	–	–	Coutu et al., (2007)

No known sequences associated with toxicity, allergenicity or pathogenicity proteins were used in creating the safflower events presented in this application.

ORDER:	Asterales
FAMILY:	Asteraceae
GENUS:	*Carthamus*
SPECIES:	*C. tinctorius*
COMMON NAME:	Safflower

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

Detailed overviews of the origin and cultivation of safflower have been published (for example: Oelke et al., 1992; Jochinke et al., 2008; OGTR 2015).

Historically safflower was grown for the flowers or floral pigments that were used in making red (carthamin), orange and yellow dyes for colouring fabrics (Dajue and Mündel 1996). Use dates back to ancient Egyptian times with references to use in the pigmentation of textiles. Globally, the current primary use for safflower is edible seed oil for use in cooking, salad oils and margarine and as a birdseed. The meal left over after extraction of oils from seeds can be also used as a stockfeed for cattle and other livestock, although meal quality is considered inferior for monogastric animals. This is because the seed hulls are high in fibre and not easily digested (Dajue and Mündel 1996). Other uses include safflower flowers used in cooking as a cheaper substitute for saffron, medical applications, the oil of a GM safflower has been approved by the US-FDA for use as a dietary supplement (Nykiforuk et al. 2012) and more recently industrial applications. Monounsaturated fatty acids such as oleic acid are highly heat stable and biodegradable and are well suited to use in the oleochemical industry (bio-based plastics, foams, and fluids) and could replace petroleum based sources in the manufacture of a number of industrial products such as lubricants and hydraulic fluids (GRDC 2010). Other minor industrial uses for safflower oil include cosmetics, soaps, and infant formula.

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

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

See Section A.2(a) (ii) above.

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

See Section A.2(a) (ii) above.

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

The intended market for Event 26 and Event 40 SHOSO is the oleochemical market.

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

No specific processing is required to make safflower seed, meal or oil safe to eat.

A.3. The nature of the genetic modification

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

The safflower advanced breeding line M1582 was transformed with *Agrobacterim tumefaciens* following the method of Belide et al., 2011 (Figure 2). Transformation introduced DNA sequences from the vector pCW732 containing a cassette intended to down regulate within the seed Δ12 desaturase (*CtFAD2.2*) and palmitoyl-ACP thioesterase (*CtFATB*) through the mechanism of RNAi. In addition, the hygromycin phosphotransferase B gene, *hph,* under the control of a constitutive promoter sequence was introduced for *in vitro* selection.

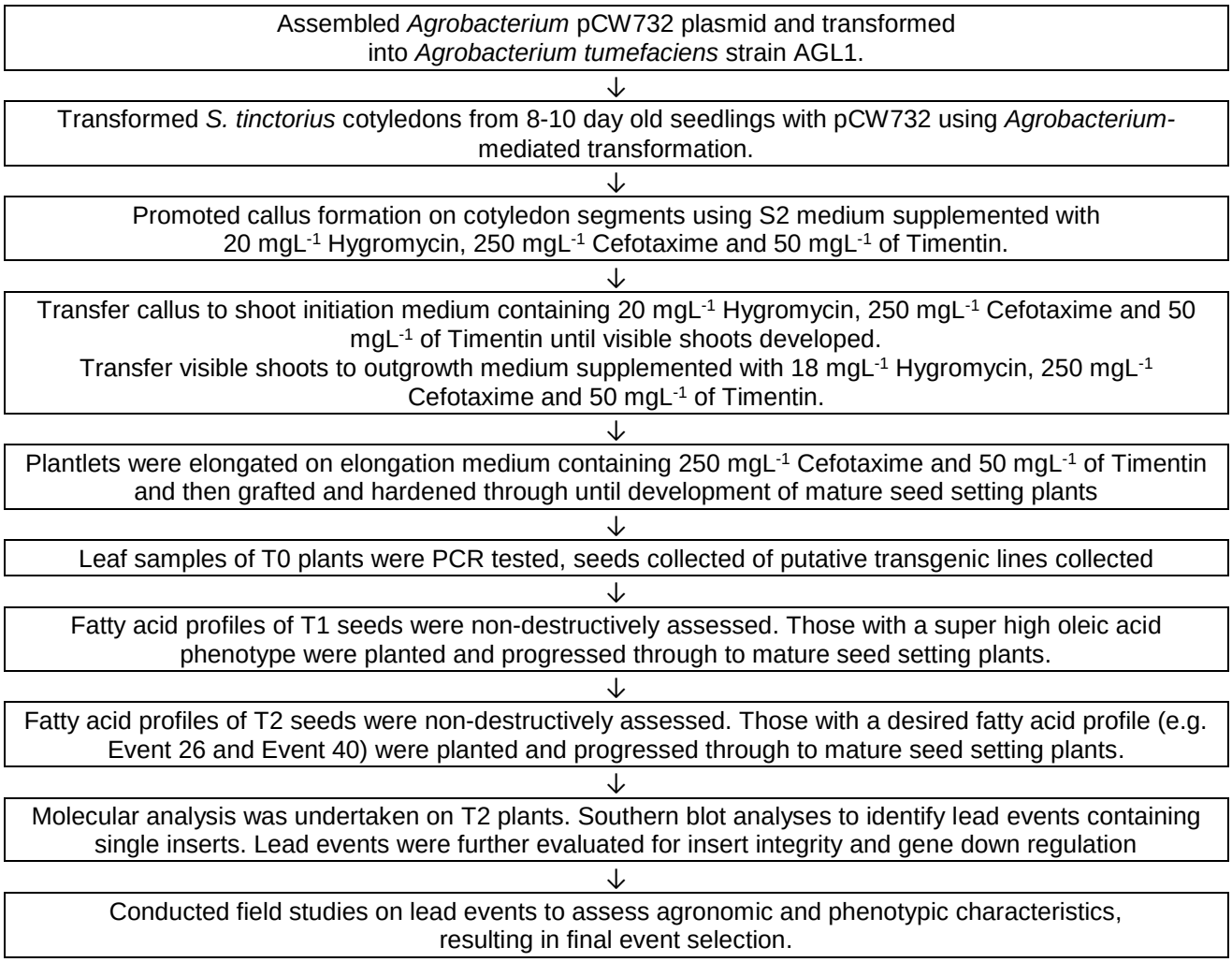

Figure 2. The development and selection of events transformed with pCW732

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

Details of the genetic material introduced into Event 26 and Event 40 are provided in Table 2. The transformation vector pCW732 (Figure 3) is within a plant binary expression vector pORE-CBIb (Coutu et al., 2007; Figure 4). The vector contains a selectable marker gene that encodes the hygromycin phosphotransferase gene (*hph*), thereby allowing selection for tolerance to hygromycin in tissue culture during the transformation process. The *hph* gene is expressed with the 35S promoter.

The pCW732 vector contains a seed specific RNAi silencing cassette using the promoter of a Flax (*Linum usitatissimum)* linin promoter (US 7,642,346) and containing a 412bp fragment of the

CtFATB gene and a 731bp fragment of the *CtFAD2-2* gene in a hairpin arrangement separated by a 742bp PDK intron sequence (int1) combined with 196bp of a Catalase 1 intron sequence (int2). The vector was constructed using the vector system described by Helliwell and Waterhouse (2005).

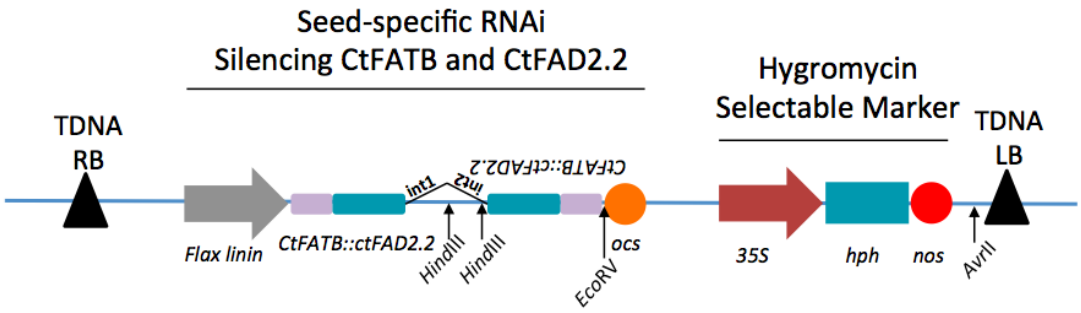

Figure 3. Schematic outline of transformation vector pCW732.

Features include: RB = Right Border; LB = Left Boarder; Flax linin = *flax linin promoter* (2032bp); CtFATB = *Carthamus tinctorius* L palmitoyl-ACP thioesterase (412bp); CtFAD2.2 = *Carthamus tinctorius* L. Δ12 desaturase (731bp); int1 = PDK intron sequence (742bp); int2 = Catalase 1 intron sequence (196bp); ocs = octopine synthase polyA (743bp); nos = nopoline synthase polyA (253bp); 35S = 35S promoter (462bp); hph = hygromycin phosphotransferase gene (1237bp); *Hind*III, *Eco*RV and *Avr*II are restriction sites.

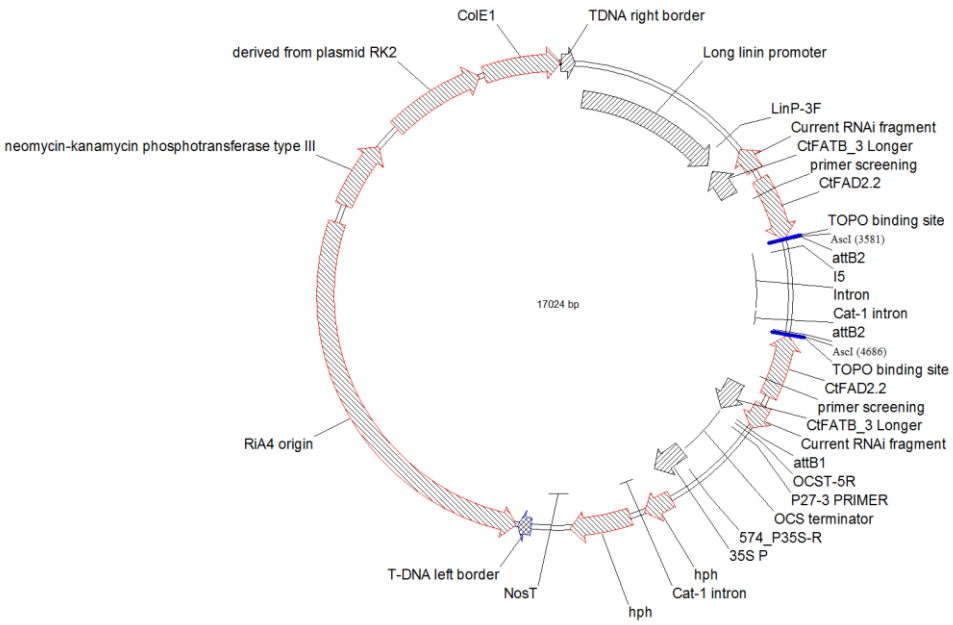

Figure 4. Binary vector pORE-CBIb containing the down regulation cassette.

The binary transformation vector pCW732 contains the backbone of pORE-CBIb (after Coutu et al., 2007). The pCW732 transformation vector was used to introduce the *CtFATB* and *CtFAD2.2* down regulation cassette into the safflower breeding line M1582.

Safflower Genes Targeted for Down Regulation using RNAi

Transcription of the inverted repeats leads to down regulation of endogenous target genes through production of dsRNA and the plant's RNAi pathway. The inverted repeats are derived from the DNA sequences of two target safflower genes (*CtFAD2.2 and CtFATB*; Table 1).

The T-DNA in the pCW732 plasmid contains down-regulation cassettes that result in the production of siRNA in Event 26 and Event 40 using the plant's RNAi pathway. As described above, each down-regulation cassette is comprised of DNA sequence arranged as an inverted repeat.

Due to the nature of the inverted repeat sequences, their transcripts form dsRNA through complementary binding. The dsRNA act as a precursor for the plant's own RNAi post-transcriptional regulatory pathway. A cellular RNase III enzyme, Dicer, recognises and processes the precursor dsRNA into 21-24 bp duplexes termed siRNA. The siRNA bind with cellular proteins forming RNA Induced Silencing Complexes (RISC). The RISC selectively degrades one of the siRNA strands, referred to as the passenger strand. The remaining strand, referred to as the guide strand, targets the complementary sequence in an mRNA molecule. Once the guide strand pairs with an mRNA in a RISC complex, the mRNA molecule is cleaved and degraded, preventing protein translation (Chau and Lee, 2007; Fire et al., 1998).

Gene for antibiotic selection

Genes encoding resistance to antibiotics are a common type of selectable marker in GM plants. The *hph* gene confers resistance to the antibiotic hygromycin B. *Hph* genes have been isolated from *E.coli* (also referred to as the *aph(4*) gene) and *Streptomyces hygroscopicus* (*aph(7)*) (Kuhstoss and Rao 1983; Leboul and Davies 1982; Rao et al. 1983). The encoded hygromycin phosphotransferase (HPH) enzymes inactivate hygromycin B (Pardo et al. 1985; Rao et al. 1983).

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

A.3(c)(i) Identification of all transferred genetic material and whether it has undergone any rearrangements

Structure of the pCW732 Inserts in Event 26 and Event 40

Donor DNA in the insert consists of both coding and non-coding genetic elements from the plasmid pCW732 as described in Table 2.

Genome walking was used to characterise the insertion of the pCW732 cassette and identify sequences upstream and downstream of the insertion. In both cases, results support a single copy, single locus insertion.

Although Southern blot analysis can provide some details with regard to the number of T-DNA insertion events in a transgenic line, it is unable to provide any precise information regarding the exact location of the T-DNA insertion within the genome. A PCR-based genome walking analysis was conducted on DNA extracted from Event 26 and Event 40 plants. Genome walking approaches are also able to identify regions of the genome adjoining the T-DNA left and right border sequences. The approach is considered unbiased as the only prior information required is for the rationale design of primers homologous to the T-DNA borders, but there is no prior knowledge needed for the reverse adapter/primer combination.

Flanking sequences identified from genome walking were cloned, sequenced and compared within an internal CSIRO safflower genome database. The structure of each insertion is provided below.

PCR based genome walking analysis

Materials and methods

High quality DNA isolated from Event 26 and Event 40 plants (T4 and T7) was used to fine map the precise location of T-DNA location in the safflower genome. A genome walking kit (the Universal GenomeWalker™ 2.0 kit (Clontech) and associated protocols) was used to find flanking sequences outside of the T-DNA Left and Right borders (Figure 5). The approach uses a restriction digest of plant genomic DNA, followed by adaptor ligation and PCR-based amplification to allow cloning of the resulting fragments and subsequent sequencing. The design of the primer+adaptor pair is located near the Left Border or Right Border sequence, respectively, of the T-DNA so that sequences flanking the T-DNA are amplified. For the Left border reaction DNA was digested Dra1. For the Right Border analysis the DNA was digested with EcoRV.

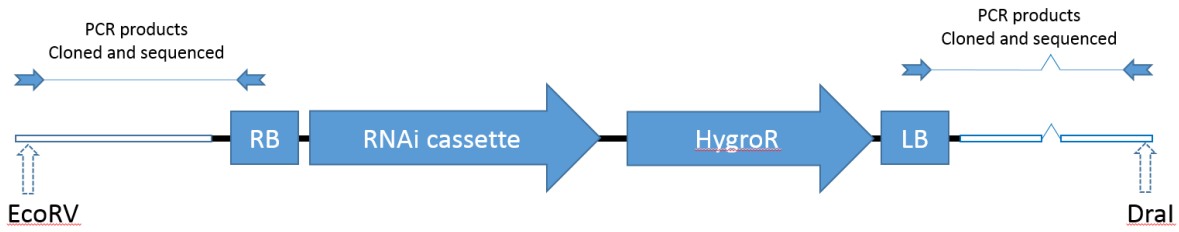

Figure 5. Schematic of PCR based genome walking analysis of Event 26 and Event 40.

Results for Event 26

Genome walking analysis of DNA isolated from both T4 and T7 Event 26 plants using an adaptor / primers pair for regions flanking the Left Border found only one amplicon of approximately 1000 bp long (Figure 6). The analysis using an adaptor/primer annealing regions flanking the Right Border also found only one amplicon, approximately 1400 bp long (Figure 6). The sequences flanking the Left and Right Border were cloned and sequenced and were found to reside on the same DNA fragment within a CSIRO safflower genome database.

Results for Event 40

Genome walking analysis of DNA isolated from both T4 and T7 Event 40 plants using an adaptor / primers pair for regions flanking the Left Border found only one amplicon of approximately 1000 bp long (Figure 7). The analysis using an adaptor/primer annealing regions flanking the Right Border also found only one amplicon, approximately 6000 bp long (Figure 7). The sequences flanking the Left and Right Border were cloned and sequenced and were found to reside on the same DNA fragment within a CSIRO safflower genome database.

Conclusion

Overall, these results are consistent with Event 26 and Event 40 containing a single copy complete T-DNA insertion with no other partial T-DNA components in the genome. The same results from both Left Border and Right Border analyses were generated from the DNA of plants from both T4 and the T7 generation, and are indicative of a stable genome arrangement.

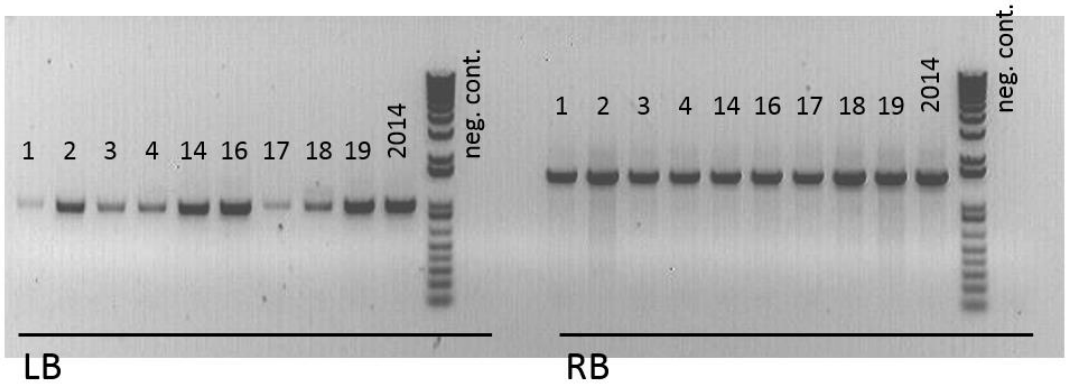

Figure 6. PCR-based genome walking for Event 26 plants from the T4 and T7 generation.

Left Border (LB) and Right Border (RB) genome walking PCR fragments amplified from DNA isolated from field grown Event 26 plants. 2014: amplified from DNA obtained from a field grown T4 plant in 2014; Independent lines 1, 2, 3, 4, 14, 16,17, 19 amplified from DNA obtained from field grown T7 plants in 2016. The lanes labelled 'neg' are a negative control where safflower DNA was replaced with water in the protocol.

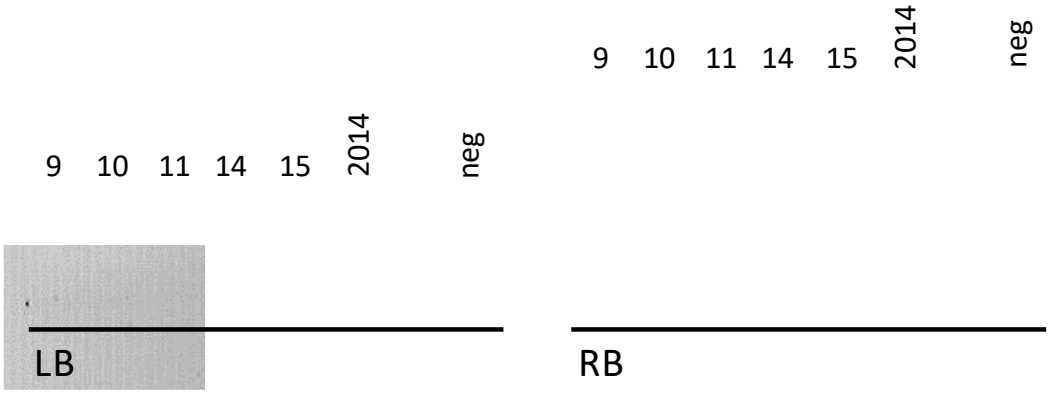

Figure 7. PCR-based genome walking for Event 40 plants from the T4 and T7 generation.

Left Border (LB) and Right Border (RB) genome walking PCR fragments amplified from DNA isolated from field grown Event 40 plants. 2014: amplified from DNA obtained from a field grown T4 plant in 2014; Independent lines 9, 10, 11, 14 and 15 amplified from DNA obtained from field grown T7 plants in 2016. The lanes labelled 'neg' are a negative control where safflower DNA was replaced with water in the protocol.

Precise genome arrangements at the region of insertion of T-DNA for Event 26 and Event 40

The amplicons identified from PCR-based genome walking were cloned and sequenced to determine the exact DNA sequence flanking the site of T-DNA insertion for Event 26 and Event 40. Sequences were aligned to an in-house CSIRO safflower genome database and the genome sequence as published by Bowers et al. 2016.

Materials and methods

Amplicons from the genome walking analysis were cloned and sequenced using standard techniques (Sanger sequencing, BigDye Chemistry). Sequences flanking both the Left Border and Right Border of T-DNA site of insertion for Event 26 and Event these were used as a query sequence into a CSIRO draft DNA assembly (unpublished). The assembly of the draft safflower genome was based on an Illumina-based short read sequencing datasets on safflower (commercial supplier), and these read sets were assembled into a draft genome using bioKanga software (CSIRO

software; unpublished). This draft genome covers approximately 80% of the safflower genome and in approximately 200,000 fragments/contigs. The flanking sequences for Event 26 were also aligned with a recent genome database of safflower (Bowers et al., 2016).

Results for Event 26

The sequences flanking the Left and Right Border for Event 26 (Figure 6) were cloned and sequenced and found to align to a single DNA sequence generated via next generation sequencing methods on a wild type non-GM safflower genome. The combination of sequencing of the Event 26 amplicons and the alignment to the wild type safflower genome found that the insertion of T-DNA generated a 69 bp deletion within the genomic region (Figure 8). Further, the analysis revealed that the entire Left Border sequence (160bp) was inserted into the genome, but only 41 bp of the162 bp Right Border sequence was inserted.

Sequence analysis also revealed that 191 bp of the replication of origin RiA4 from the binary vector pORE-CBIb was also inserted.

Based on this analysis a map of the pCW732 insertion into Event 26 was generated (Figure 9).

THIS FIGURE IS COMMERCIALLY SENSITIVE

Figure 8. Sequence characterisation of the T-DNA insertion of Event 26.

Sequenced amplicons identified from PCR-based genome walking analysis (E26_RB Junction and E26_LB Junction) were aligned to the sequences of the pCW732 vector (pCW732_LB and pCW732_RB) and a CSIRO safflower database (Bower16_13860-7_8). **INFORMATION IN THIS FIGURE IS COMMERCIALLY SENSITIVE**

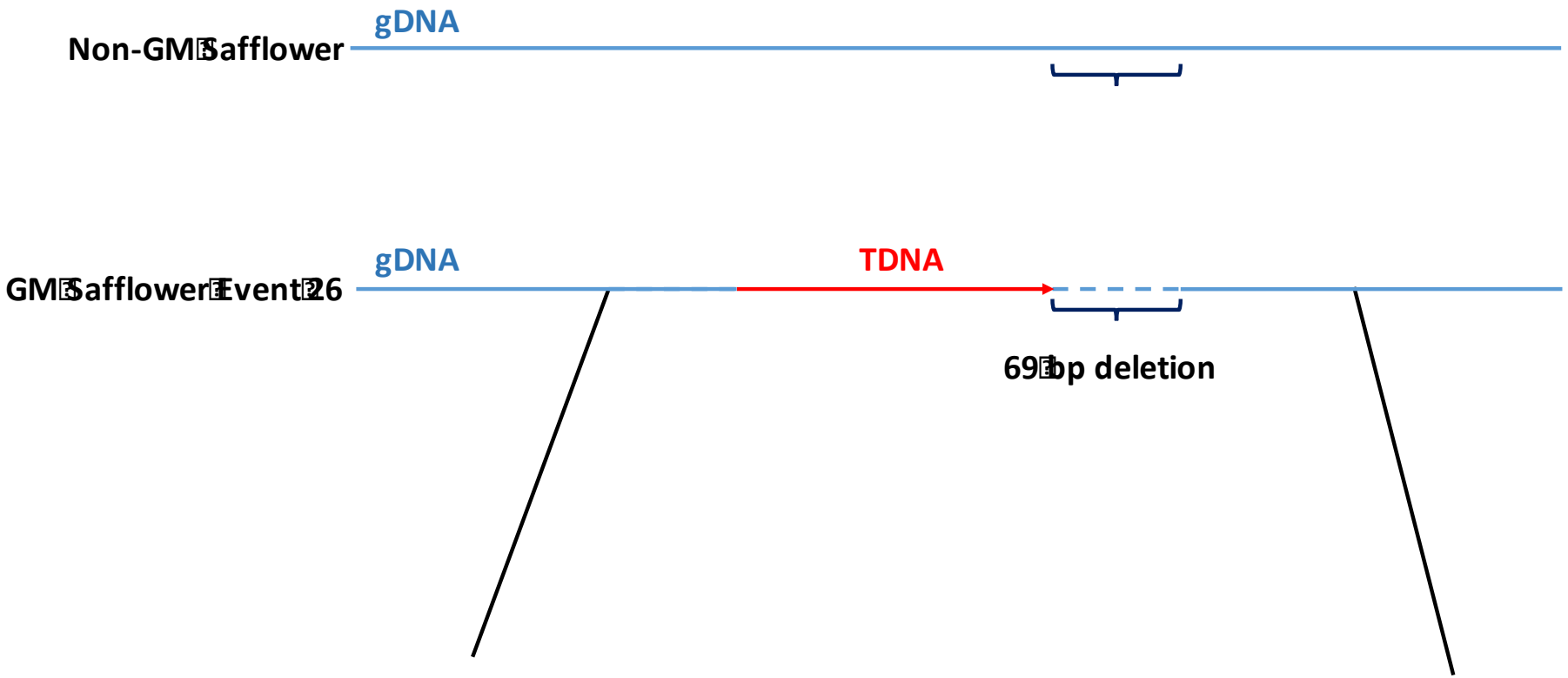

Figure 9. Schematic structure of the Event 26 insertion of pCW732 into the safflower line M1582.

The schematic map shows 1000 bp upstream and downstream of the insertion site, position of a deletion and the position of the elements of the T-DNA sequences that remain after insertion. E26TF and E26GR are the priming sites for an Event 26 specific PCR. Details of the pCW732 elements are provided in Table 1.

Results for Event 40

The sequences flanking the Left and Right Border for Event 40 (Figure 7) were cloned and sequenced and found to align to a single DNA sequence generated via next generation sequencing methods on a wild type non-GM safflower genome. The combination of sequencing of the Event 40 amplicons and the alignment to the wild type safflower genome found that the insertion of T-DNA generated a 34 bp deletion and 35 bp duplication within the genomic region (Figure 10). Further, the analysis revealed that both the Left Border (16bp) and Right Border (39 bp) sequences were truncated.

Based on this analysis a map of the pCW732 insertion into Event 40 was generated (Figure 11).

THIS FIGURE IS COMMERCIALLY SENSITIVE

Figure 10. Sequence characterisation of the T-DNA insertion of Event 40.

Sequenced amplicons identified from PCR-based genome walking analysis (E40_RB Junction and E40_LB Junction) were aligned to the sequences of the pCW732 vector (pCW732_LB and pCW732_RB) and a CSIRO safflower database (gx_s317 Scaff097804). **INFORMATION IN THIS FIGURE IS COMMERCIALLY SENSITIVE**

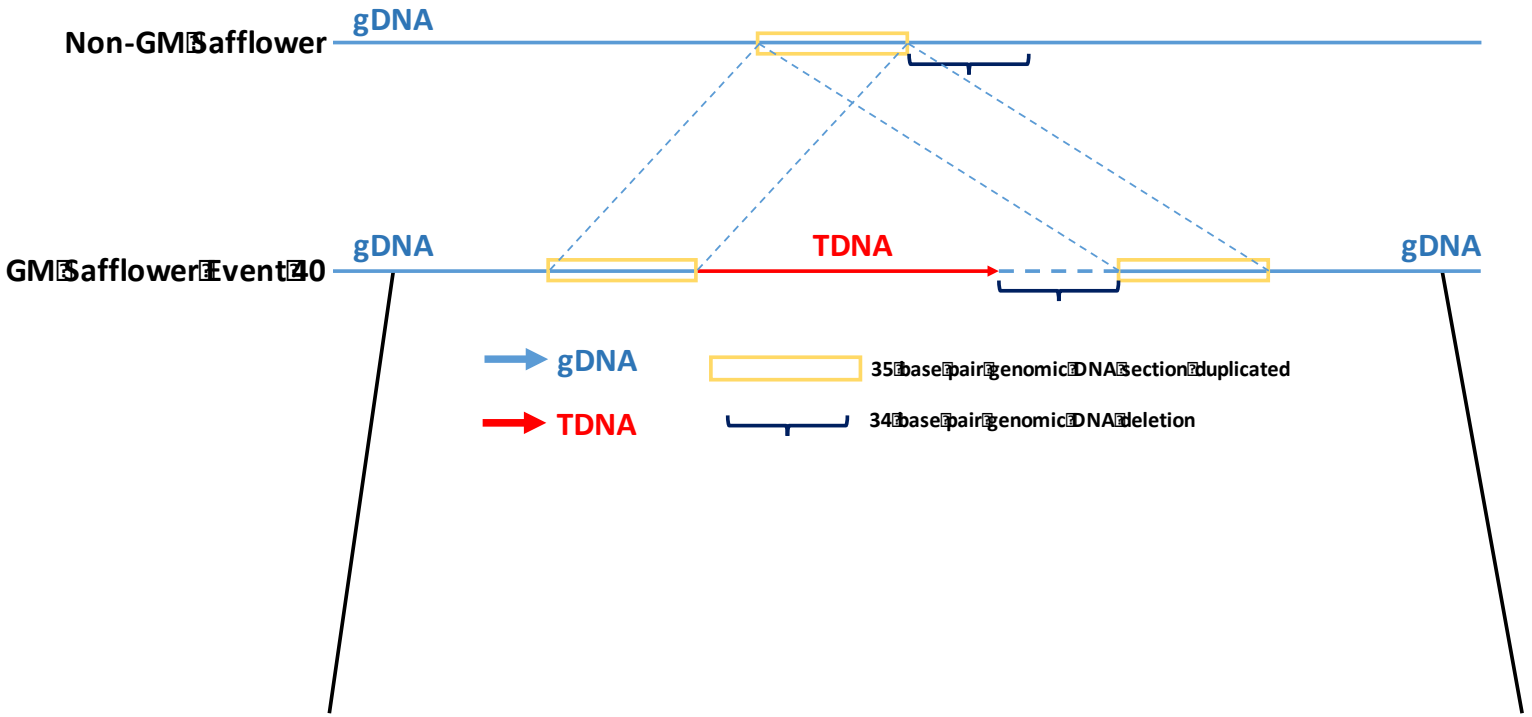

Figure 11. Schematic structure of the Event 40 insertion of pCW732 into the safflower line M1582.

The schematic map shows 1000 bp upstream and 960 downstream of the insertion site, duplications, position of a deletion and duplication and the position of the elements of the T-DNA sequences that remain after insertion. E40GF and E40GR are the priming sites of the genome walking analysis. Note that a deletion of 34 bases was also found at the site of the 35 bp duplication. E40_TF and E40_GR2 are priming sites for Event 40 specific PCR. Details of the pCW732 elements are provided in Table 1.

Conclusions

Molecular characterisation of the insertion sites for Event 26 and Event 40 support a single intact copy of the pCW732 cassette. Insertions resulted in only small changes in the safflower genome.

A.3(c)(ii) A determination of the number of insertion sites, and the number of copies at each insertion site

The number of loci associated with the insert from pCW732 in a number of independent Events (including Event 26 and Event 40) was assessed using Southern blot analysis. DNA from transgenic safflower and a non-GM safflower (variety S-317) were isolated and digested with either the *Kpn*I or *Pac*I restriction enzymes and hybridised with a probe for the hygromycin phosphotransferase gene. Both *Kpn*I or *Pac*I enzymes digest within the T-DNA fragment, but outside of the hygromycin region (Figure 12). Therefore, this strategy can determine the presence of sequences annealing to the hygromycin probe and therefore also provide an estimate of the number of T-DNA insertions in the Event.

PCR analysis was undertaken on safflower DNA isolated from Event 26 and Event 40. The results suggest that no vector backbone sequences were inserted during the transformation process.

Southern Blot Analysis

Methods

Ten plants of Event 26 and Event 40 (T4 generation) were grown under glasshouse conditions. For each Event, plant material was combined to prepare high quality DNA suitable for enzymatic restriction digestion with either of *Kpn*I or *Pac*I enzymes.

Southern blot analysis was conducted following a protocol as previously published (Belide et al., 2011). High quality DNA from safflower was isolated using caesium chloride gradients. Generally, 1 mg of DNA was used for overnight digestion using High Fidelity enzymes, *Knp*I or *Pac*I (NEB, USA). For preparation of the probe, PCR primers were used to amplify the entire coding region of the *Hph* gene from pCW732. This Amplicon was used as a template for a further round of PCR to generate a clean template for the probe free of contaminating vector backbone sequence. The probe was radiolabelled using random primer integration and radiolabelled P^{32}-NTP nucleotides, as previously described (Belide et al., 2011).

Band size determination

A DNA ladder was not included on safflower Southern blots. This was for two reasons:

1. Molecular size ladders probed with P^{32}-NTP nucleotides can often provide signals that dominate blots making it difficult to observe the restriction patterns of test lines.
2. There is a tendency for bands above about 3 kb to migrate faster than similarly-sized molecular weight markers within the same gel. The observed faster migration appears to be due to residual polysaccharides often present in plant genomic DNA samples. The molecular weight markers run faster when mixed with plant DNA samples; however, spiking the markers with plant genomic DNA to counter migration variability complicates ladder interpretation because of the potential hybridisation to endogenous DNA that may occur. This discrepancy between band and marker migration makes it often difficult to accurately determine sizes for unknown bands, and impacts the observed sizes of expected bands.

A schematic diagram of a molecular ladder is provided in Figure 13. The size of bands was estimated using a range of genome sequence resources developed by CSIRO. Performing an *in-silico* digest of the safflower genome.

Results

The Southern blot analysis determined that pCW732-26 and pCW732-40 were predicted to be single copy events (Figure 13). Other independent SHOSO Events, namely 21, 33 and 48 produced multiple bands, consistent with an interpretation of multiple T-DNA insertions within each of these Events. As expected, non-GM safflower controls produced no band or signal.

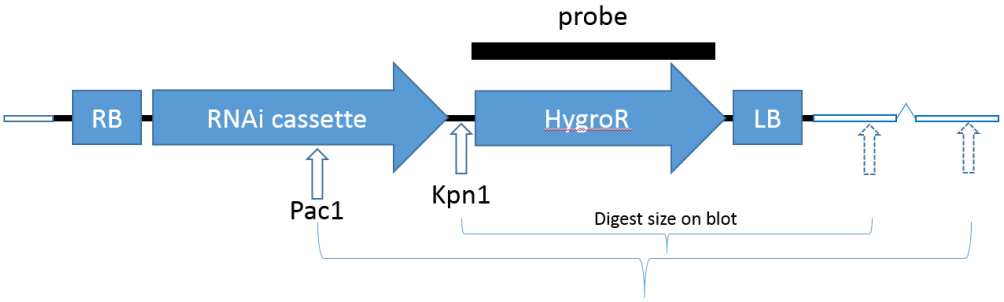

Figure 12. Schematic diagram of the pCW732 insert structure of transgenic safflower.

DNA from transgenic safflower events were digested with *Kpn*I and *Pac*I restriction enzymes and probed for the hygromycin phosphotransferase gene.

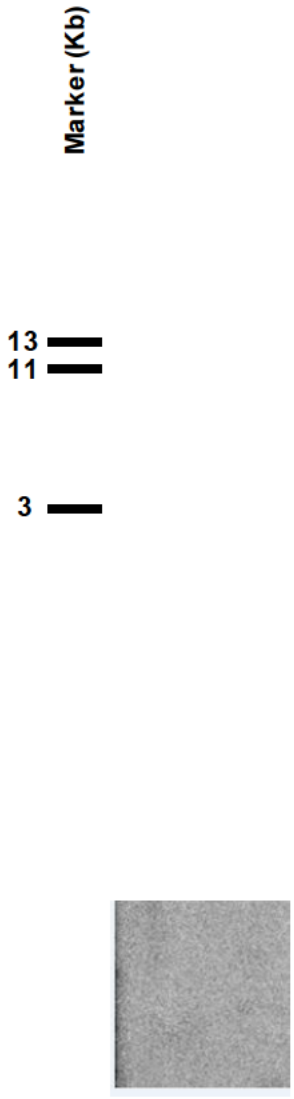

Figure 13. Southern blot analysis of safflower transformed with vector pCW732.

DNA from independent transgenic safflower lines digested with either *Kpn*I or *Pac*I and probed with a fragment aligning with the hygromycin phosphotransferase gene. The lane markers from left to right include DNA digested with *Kpn*I enzyme, far left S-317 as a non-GM negative control; pCW732-40 (Event 40); pCW732-48 (Event 48); pCW732-22 (Event 22); pCW732-21 (Event 21); pCW732-21 (Event 21, repeated); pCW732-26 (Event 26); pCW732-30 (Event 30); pCW732-34 (Event 34); pCW732-33 (Event 33). The same sequence of samples is repeated although the DNA was digested with *Pac*I, namely S-317 as a non-GM negative control; pCW732-40 (Event 40); pCW732-48 (Event 48); pCW732-22 (Event 22); pCW732-21 (Event 21); pCW732-21 (Event 21, repeated); pCW732-26 (Event 26); pCW732-30 (Event 30); pCW732-34 (Event 34); pCW732-33 (Event 33) at the far-right hand side of the panel. Fragment sizes were estimated from *in silico* digests.

Analysis of Event 26 and Event 40 for the presence of vector backbone

During the integration of T-DNA into the safflower genome there is a potential for different fragments of the binary vector to also insert into the plant genome. One way to assess for insertion of T-DNA vector backbone is to undertake PCR analysis using a range of primers that span the regions of the vector that lie outside of the T-DNA region. PCR analysis was undertaken on safflower DNA isolated from Event 26 and Event 40. The results suggest that no vector backbone sequences were inserted during the transformation process.

Materials and methods

Five pairs of PCR primers were designed across regions outside of the T-DNA, including regions of homology to the bacterial origin of replication and the bacterial chemical selection marker *Npt*II (see Figure 4 and Figure 14A).

DNA was isolated from plants at the T6 generation for Event 26 and Event 40 grown in the field under DIR131 and subjected to PCR using standard molecular biology techniques. PCR products were separated on 1% Agarose gels.

Non-GM safflower (variety S-317) was used as a negative control and plasmid DNA from binary vector pCW732 was used as a positive control. All controls were tested demonstrating detection of target fragments. This included presence of vector spiked into control DNA (Figure 14B)

P1FW	5'–GTC GGC AAA TCG TCA GAC TT–3'
P1RV	5'–GTG GAG TCA GGC TTG ATC GT–3'
P2FW	5'–TGT TCC CGG ATC GAA GTA AG–3'
P2RV	5'–ACA TCC TTG GCG TCT CAA CT–3'
P3FW	5'–AAT GTT CGA ATG CCC TTC TC–3'
P3RV	5'–AGG GCG ACC TCT TTT TGG–3'
P4FW	5'–GAC AAG TGG TAT GAC ATT GC–3'
P4RV	5'–CTA AAA CAA TTC ATC CAG TA–3'
P5FW	5'–AGA TCC TCT TCC GCT TCC TC–3'
P5RV	5'–TAC CGG GTT GGA CTC AAG AC–3'

Results

There were no detectable PCR products in DNA samples from either Event 26 or Event 40 (Figure 14C).

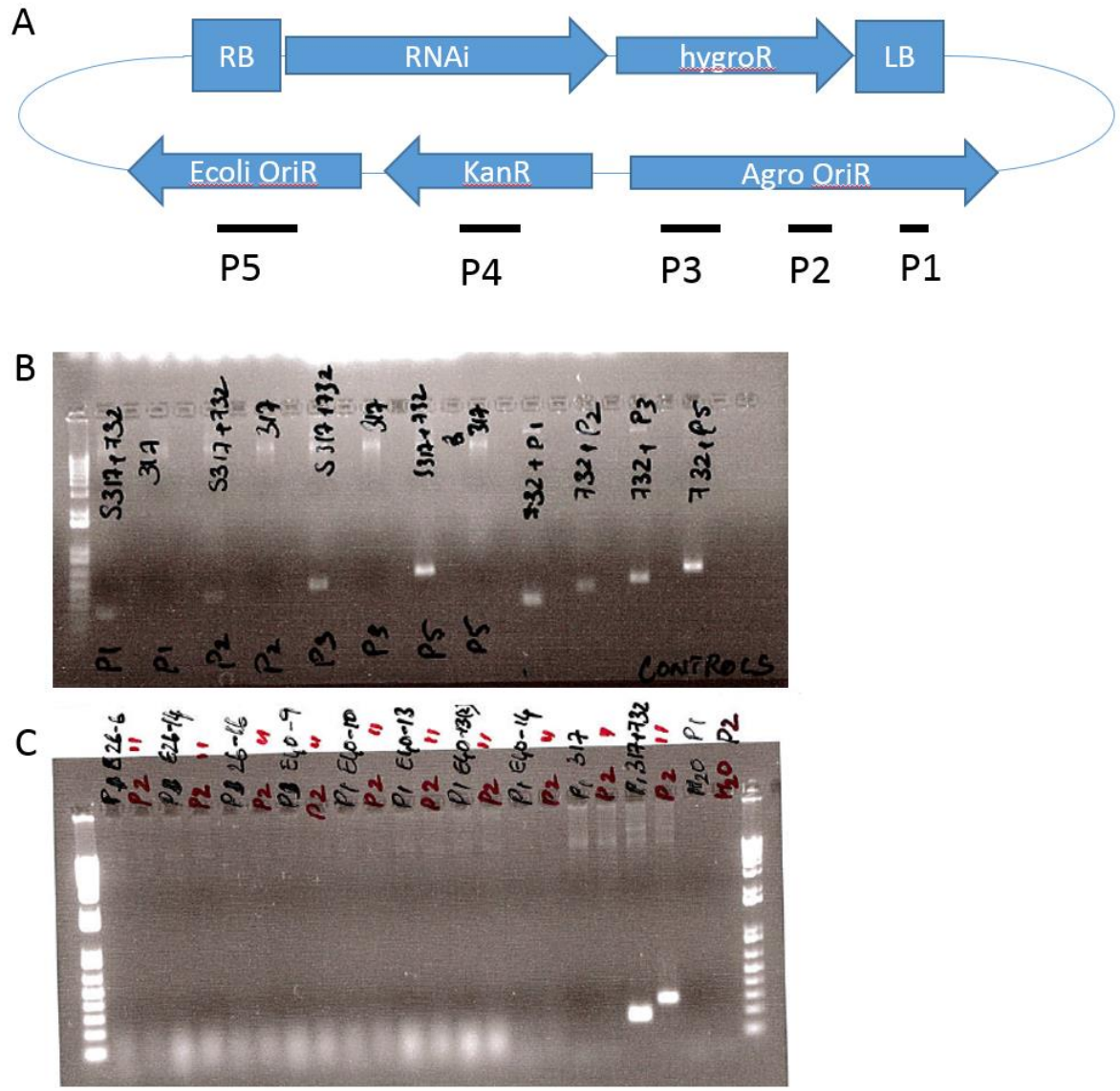

Figure 14. PCR-based analysis of vector backbone contamination in Event 40 and Event 26.

Panel A: Placement of primers across the T-DNA backbone of vector pCW732; Panel B: verification of positive and negative PCR for the primer sets across the pCW732 backbone. Note the different lengths for each amplicon product. The intact and complete vector pCW732 was spiked into S317 safflower samples to demonstrate a positive result; Panel C: An example of the absence of vector contamination in independent DNA samples from field grown Event 26 and Event 40 plants. In all PCR assay runs, positive controls were used to generate amplicons as a reference.

A.3(c)(iii) Full DNA sequence of each insertion site, including junction regions with the host DNA

The full DNA sequence of Event 26 and Event 40 are provided in Appendix 1.

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

See Figure 9 for Event 26 and Figure 11 for Event 40.

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

An analysis was completed to assess open reading frames (ORFs) in the Super High Oleic Acid Safflower Event 26 and Event 40, which were generated by transformation of the high Oleic acid breeding line M1582 with pCW732 T-DNA. Novel ORFs were evaluated using bioinformatics (i.e., amino acid sequence comparison techniques) to assess their similarity to known toxins or allergens.

Bioinformatic analyses of 65 start-to-stop ORFs within the DNA insert and genomic junction regions of Event 26 and 62 start-to-stop ORFs within the DNA insert and genomic junction regions of Event 40, demonstrated no putative polypeptides with relevant homology to proteins that are known to be toxic, allergenic, or to have other biologically adverse properties.

Objectives and methodology

An analysis was completed to determine the toxin or allergen homology, if any, of open reading frames (ORFs) introduced into Event 26 and Event 40 through transformation with pCW732 T-DNA. This report assesses the allergen and toxin homology of the start-to-stop open reading frames in the Event 26 and Event 40 insert and junction regions using the standard bioinformatic techniques summarised in Table 3.

Table 3. Overview of analyses using bioinformatics

Analysis	Purpose	Approach
Start-to-stop ORF Analysis	Identify all open reading frames associated with the T-DNA insert, including junction regions	pCW732 insert and at least 1000bp of M1582 flanking sequence analysed in SnapGene that systematically identifies all ORFs (≥30 amino acids) located between a start codon and a stop codon where all six reading frames are considered
Allergenicity Analysis	Ensure that known allergenic sequences have not been introduced through transformation of pCW732	AllergenOnline (FASTA Search): identify any small regions of similarity or larger regions of homology between ORFs and known allergens
Toxicity Analysis	Ensure that sequences similar to known toxins have not been introduced through transformation of pCW732	FASTA Search (fasta36): Identify any annotated toxins with regions of similarity to ORFs associated with the insert

Expression of toxins or allergens is unlikely, particularly in safflower transformed with safflower DNA for the down regulation of faty acid biosynthesis genes *CtFATB* and *CtFAD2.2*. However, the left and right junctions of the Event 26 and Event 40 inserts have potential to express ORFs due to the novel junctions that could be transcribed by regulatory sequences located in the nearby safflower genome.

Well-established techniques in bioinformatics were used to evaluate the inserts in Event 26 and Event 40 (Ladics, 2007; Goodman, 2008; Terrat, 2013).

This study aimed to assess the allergen and toxin homology of the start-to-stop open reading frames in the Event 26 and Event 40 insert and junction regions using the standard bioinformatic techniques. The study had 3 primary objectives:

1. Perform a comprehensive start-to-stop ORF analysis covering the DNA insert and its genomic junctions with the flanking regions.
2. Identify and evaluate any potential allergens based upon similarity between the ORFs and known allergens; and
3. Identify and evaluate any potential toxins based upon similarity between the ORFs and known toxins.

ORF detection

All open reading frames (ORFs) created as a result of the pCW732 T-DNA insertion were identified using the ORF detection function within SnapGene® (version 3.3.4). The search parameters were defined to identify all ORFs with at least 30 amino acids located between the first ATG start codon in an ORF and the subsequent stop codon. Since a nucleotide sequence can be translated in three reading frames from two directions, all six reading frames of the Event 26 and Event 40 insert and flanking regions were analysed for potential ORFs. The results were converted into FASTA-formatted files containing all unique protein sequences for further analysis.

Allergenicity database searches

Allergenicity potential was evaluated using the public, allergen-specific search engine (http://www.allergenonline.org/databasefasta.shtml) available through the Food Allergy Research and Resource Program (FARRP) at the University of Nebraska. All searches were performed using the most current database (version 17; January 18, 2017). Version 17 contains 2035 protein sequence entries that are categorised into 808 taxonomic-protein groups of unique proven or putative allergens (food, airway, venom/salivary and contact). Some of the allergenic wheat gliadins or glutenins may also cause celiac disease, however they are listed on the allergen site if there is evidence of IgE binding. The ORFs were analysed using full-length, 80-mer local, and 8-mer exact match alignments.

Toxin database searches

The most recent UniProt database (http://www.uniprot.org) 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 $1x10^{-4}$:

ORF identification and analysis

Start-to-stop ORF identification in Event 26

An open reading frame is a contiguous sequence located between a canonical start codon and the next in-frame downstream stop codon. SnapGene® (version 3.3.4) was used to identify all start-to-stop ORFs contained in the T-DNA insert and the adjacent M1582 safflower flanking sequence, as shown schematically in Figure 15.

For Event 26 there were 64 unique ORFs identified, which were used in the subsequent allergen and toxin analyses.

Start-to-stop ORF identification in Event 40

An open reading frame is a contiguous sequence located between a canonical start codon and the next in-frame downstream stop codon. SnapGene® (version 3.3.4) was used to identify all start-to-stop ORFs contained in the T-DNA insert and the adjacent M1582 safflower flanking sequence, as shown schematically in Figure 16.

For Event 40 there were 62 unique ORFs identified, which were used in the subsequent allergen and toxin analyses.

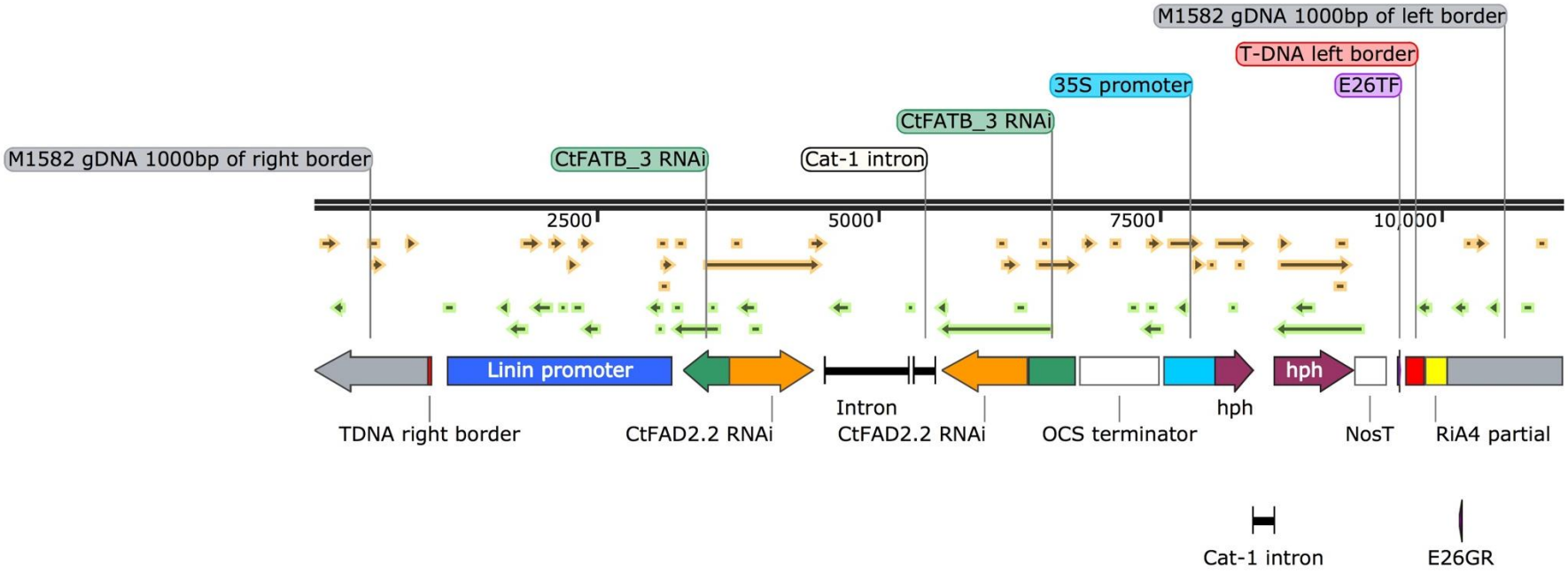

Figure 15. Complete ORF analysis for Event 26

A schematic diagram illustrating any T-DNA insertion site in the plant genome of Event 26. All ORFs ≥30 amino acids (coloured arrows) contained within the DNA insert or overlapping the genomic flanking sites on either end are identified and were used for subsequent analysis.

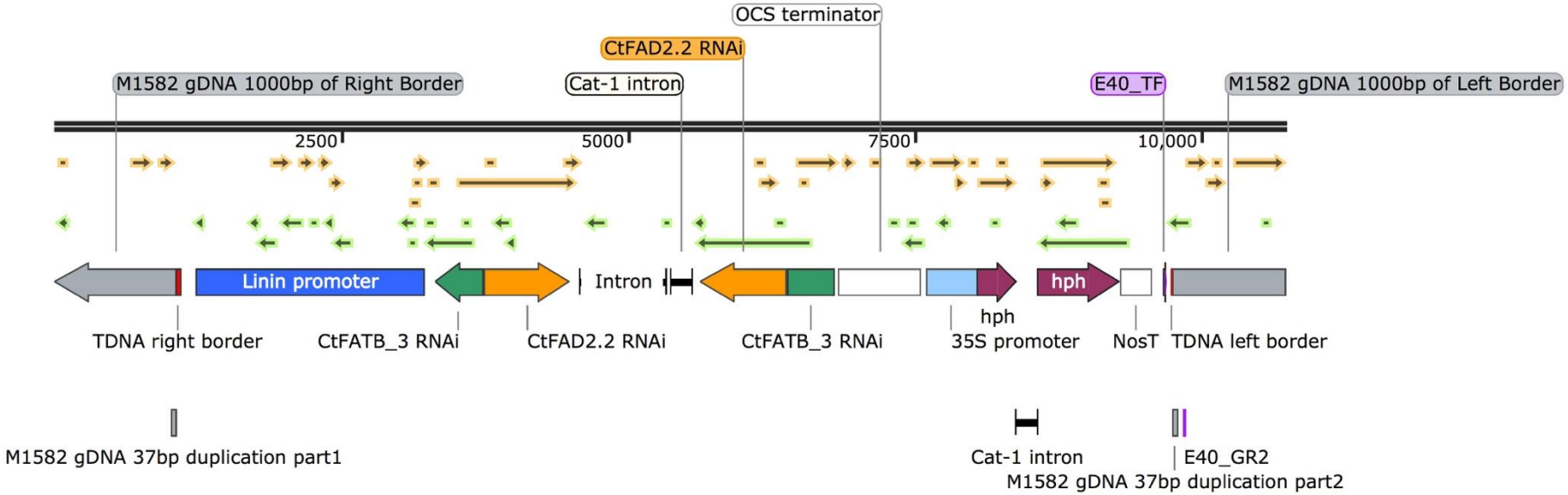

Figure 16. Complete ORF analysis for Event 40

A schematic diagram illustrating any T-DNA insertion site in the plant genome of Event 40. All ORFs ≥30 amino acids (coloured arrows) contained within the DNA insert or overlapping the genomic flanking sites on either end are identified and were used for subsequent analysis.

Allergenicity assessment of Event 26

Evaluation of the 64 ORFs for similarity or identity to known allergens did not identify any significant matches. There are no allergen-based safety concerns associated with the Event 26 insert, including ORFs extending into flanking sequences.

The allergenic potential of the ORFs was assessed using the web-based tool (http://www.allergenonline.org/databasefasta.shtml) provided by the Food Allergy Research and Resource Program (FARRP). This tool allowed comparison of ORFs associated with the Event 40 insert to known allergens using multiple sequence identity searches:

1. full-length alignments.
2. 80 amino acid (80-mer) high local-identity alignments; and
3. 8 amino acid (8-mer) exact matches.

Full-length matches consisting of greater than 50% identity are considered the most predictive of potential allergens (Aalberse, 2000), whereas the 80-mer search is a precautionary tool to identify smaller regions of high identity between any ORFs and those of known allergens. The allergen database contains the sequence of known allergens, but the specific sequence responsible for allergenicity is not necessarily known, nor is it known whether an 8-mer is capable of inducing an allergic response (Goodman, 2008).

Full-length homology search

A full-length homology search was performed to identify homology between the individual ORFs and known allergens. This analysis compared each of the 64 ORFs with the sequence of known allergens and reported any matches with greater than 35 % homology (E-value cut off = 10^{-4}). The algorithm did not identify any potential allergens (Table 4) associated with the Event 26 insert.

80-mer homology search

A second analysis identified localised regions of similarity between the ORFs and known allergens. This analysis compared all contiguous 80 amino acid sequences within an ORF and identified any matches with greater than 35% homology to known allergens (E-value cut off = 10). The algorithm did not identify any potential allergens (Table 4) associated with the Event 26 insert.

8-mer identity search

Lastly, a short identity analysis was performed to identify short (8-mer) regions of exact identity between ORFs and known allergens. The algorithm did not identify any potential allergens (Table 4) associated with the Event 26 insert.

Table 4. Summary of allergenicity and toxicity matches for ORFs associated with the Event 26 insertion site

Allergenicity Search Alignments			Toxicity Search Alignments
Full-length alignment	**Scanning 80-mer**	**8-mer identity**	**Full-length**
0	0	0	0

Allergenicity assessment of Event 40

Evaluation of the 62 ORFs for similarity or identity to known allergens did not identify any significant matches. There are no allergen-based safety concerns associated with the Event 40 insert, including ORFs extending into flanking sequences.

The allergenic potential of the ORFs was assessed using the web-based tool (http://www.allergenonline.org/databasefasta.shtml) provided by the Food Allergy Research and Resource Program (FARRP). This tool allowed comparison of ORFs associated with the Event 40 insert to known allergens using multiple sequence identity searches:

1. full-length alignments.
2. 80 amino acid (80-mer) high local-identity alignments; and
3. 8 amino acid (8-mer) exact matches.

Full-length matches consisting of greater than 50% identity are considered the most predictive of potential allergens (Aalberse, 2000), whereas the 80-mer search is a precautionary tool to identify smaller regions of high identity between any ORFs and those of known allergens. The allergen database contains the sequence of known allergens, but the specific sequence responsible for allergenicity is not necessarily known, nor is it known whether an 8-mer is capable of inducing an allergic response (Goodman, 2008).

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80-mer homology search

A second analysis identified localised regions of similarity between the ORFs and known allergens. This analysis compared all contiguous 80 amino acid sequences within an ORF and identified any matches with greater than 35% homology to known allergens (E-value cut off = 10). The algorithm did not identify any potential allergens (Table 5) associated with the Event 40 insert.

8-mer identity search

Lastly, a short identity analysis was performed to identify short (8-mer) regions of exact identity between ORFs and known allergens. The algorithm did not identify any potential allergens (Table 5) associated with the Event 40 insert.

Table 5. Summary of allergenicity and toxicity matches for ORFs associated with the Event 40 insertion site

Allergenicity Search Alignments			Toxicity Search Alignments
Full-length alignment	**Scanning 80-mer**	**8-mer identity**	**Full-length**
0	0	0	0

Toxicity assessment

Evaluation of the ORFs for homology to known toxins did not identify any significant matches, and indicated that there are no toxicity-based safety concerns associated with the Event 26 or Event 40 inserts.

There are no well-curated toxin-specific databases. However, the UniProt/Swissprot database (http://www.uniprot.org) is an expansive and well-annotated protein database that can be queried for protein homology. A query against the entire database, much like a query against the NCBI database, will produce many non-specific hits that must be manually and somewhat subjectively inspected to determine whether the identified proteins present toxicity safety concerns. Note, the current database contains 410,735 entries associated with the word toxin.

The ORFs associated with the Event 26 and Event 40 inserts were queried against this database using search parameters similar to the full-length allergen search at FARPP (E-value cut off, 10^{-4}). No matches were identified. A summary of the results is included in Table 4 and Table 5.

Allergen and toxin homology assessment of junction ORFs

The sequences of the ORFs covering the junctions between the inserts and the safflower flanking regions were evaluated as part of the ORF analysis. In Event 26 no ORFs were identified associated with pCW732 spanning the junction between the insert and safflower genomic DNA.

In Event 40, two ORFs associated with pCW732 spanning the junction between the insert and genomic DNA were identified. As shown above, none of the ORFs, including these junction ORFs, were identified as homologs of known toxins or allergens. The analysis did not identify a safety concern.

pCW732 junction ORFs in Event 40

There were two ORFs covering the junctions associated with the pCW732 insert in Event 40 (Table 6).

Table 6. Summary of pCW732 junction ORFs for Event 40

INFORMATION IN THIS TABLE IS COMMERCIALLY SENSITIVE

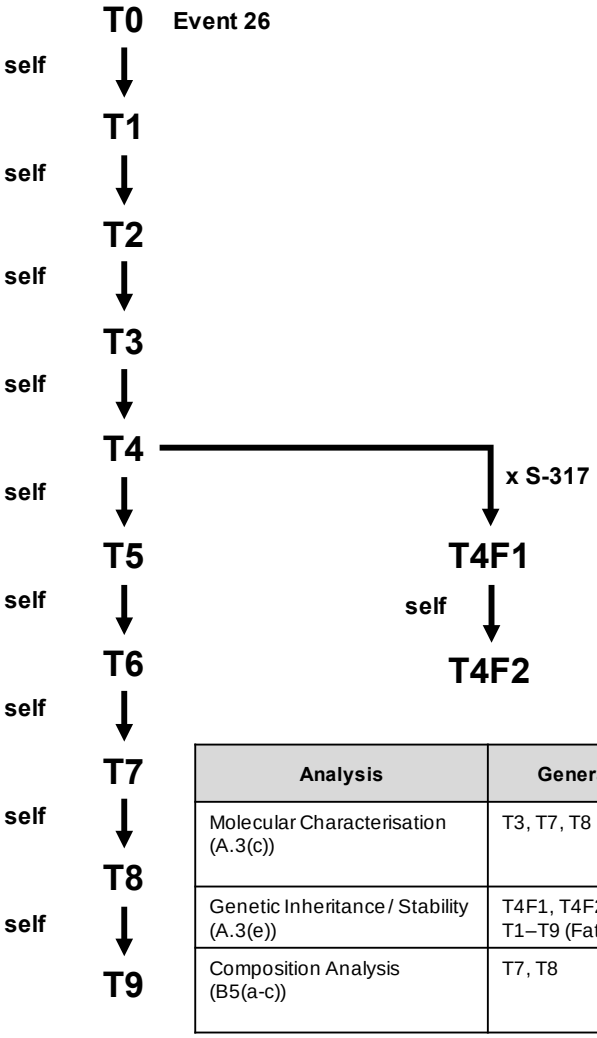
Conclusions form ORF analysis

Using well-established bioinformatic tools, a comprehensive analysis of the open reading frames in the insert and flanking regions of Event 26 and Event 40 was conducted. This bioinformatic analysis did not identify any potential toxin or allergen safety concerns with start-to-stop open reading frames associated with either of the Events.

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

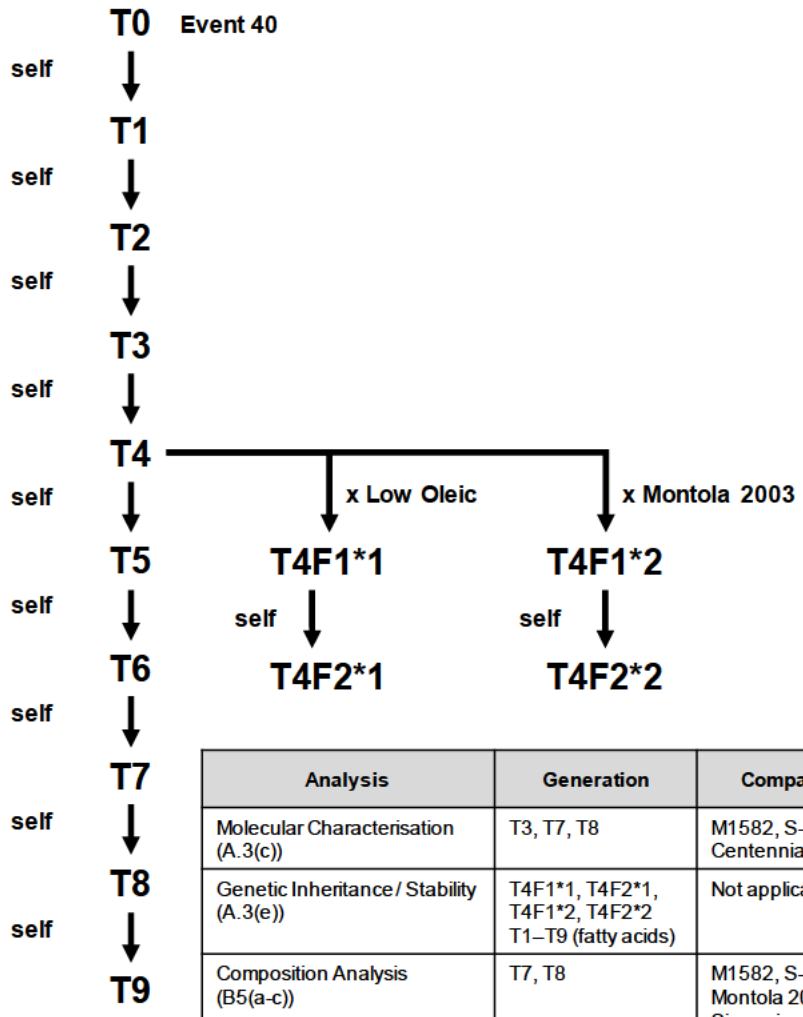
Diagrams describing the development of Event 26 and Event 40 and what generations have been used for various analysis are provided in Figure 17 and Figure 18.

Both Event 26 and Event 40 have been field evaluated over multiple environments from the T3 generation to the T9 generation. The fatty acid profiles of seed from each generation have been examined and shown to exhibit the intended phenotype.



Analysis	Generation	Comparators
Molecular Characterisation (A.3(c))	T3, T7, T8	M1582, S-317, S901, Centennial, Lesaf496
Genetic Inheritance / Stability (A.3(e))	T4F1, T4F2 T1–T9 (Fatty acids)	Not applicable
Composition Analysis (B5(a-c))	T7, T8	M1582, S-317, Montola 2003, Sironaria,

Figure 17. Diagram of the development of Event 26



Analysis	Generation	Comparators
Molecular Characterisation (A.3(c))	T3, T7, T8	M1582, S-317, S901, Centennial, Lesaf496
Genetic Inheritance / Stability (A.3(e))	T4F1*1, T4F2*1, T4F1*2, T4F2*2 T1–T9 (fatty acids)	Not applicable
Composition Analysis (B5(a-c))	T7, T8	M1582, S-317, Montola 2003, Sironaria

Figure 18. Diagram of the development of Event 40

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

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

A range of approaches were used to assess the stability of Event 26 and Event 40. Firstly, each of the Events were separately crossed into a non-GM safflower line and the inheritance pattern of the GM trait evaluated. Further, the progeny of multiple generations was tested for efficacy of the down regulation of *CtFAD2.2* and *CtFATB*, the SHOSO trait, via lipid profiling.

Crossing Event 26 with an oleic type non-GM safflower

The SHOSO Event 26 was crossed with an oleic type non-GM safflower variety S-317. Fatty acid analysis was undertaken on seed from the F2 generation. The number of F2 plants that contained levels of Oleic acid greater than 90% and Palmitic acid levels less that 3% were evaluated with respect to a 3:1 segregation ratio of the dominant SHOSO trait. Based on the analysis, the insertion of pCW732 in Event 26 is stably inherited and segregates in accordance with Mendelian inheritance.

Materials and methods

An individual plant of Event 26 (T4 generation) was grown in a glasshouse alongside a non-GM plant with a high oleic genotype (variety S-317). During flowering, the two plants were manually crossed and all unused florets were emasculated to prevent self-pollination. This procedure generated 6 forced cross seeds, termed F1 seed. The F1 seed were further grown to maturity and the flowers self-pollinated to generate a population of 119 F2 seed. Each of the 119 seed were analysed for their fatty acid profile, indicative of inheritance of the SHOSO trait.

Fatty acid analysis was undertaken on F2 safflower seeds. Firstly, the safflower seeds were imbibed on wet filter paper overnight (16 hrs) at room temperature to allow the seed coat to be easily removed. The tip of the cotyledons (5 mm) were excised and sampled for fatty acid methyl ester analysis (FAME). The remaining seed was planted in the glasshouse and grown to maturity.

FAMEs were prepared essentially as described by (Zhou et al., 2013), with slight modifications. The methylation was extended to 4 hr with scaled up to 800 µL 1N methanolic-HCl (Supelco, Bellefonte, USA). GC analysis with FID was also performed as described by (Zhou et al., 2013), except the ramping program was changed to an initial temperature at 150°C holding for 1 min, then raised to 180°C at 10°C/min, and to 240°C at 50°C/min holding for 4 min. GLC standard 411 (Nuchek, Elysain, USA) was used for calibration.

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

Results

Of the 119 F2 seed produced from the Event 26 x S-317 cross, 37 individuals had Palmitic acid levels greater than 3%. Similarly, 36 individuals had Oleic acid levels equal to or less than 90%. These results are indicative of plants that do not contain a functional Event 26 insert. Chi-square goodness of fit tests indicated that there was no significant departure from the predicted 3:1 segregation ratio (Palmitic acid greater than 3% X^2[1,N=119] = 2.36, P=0.153, p<0.01; Oleic acid levels less than 90% X^2[1,N=119] = 1.75, P=0.224, p<0.01).

Crossing Event 40 with an oleic type non-GM safflower

The SHOSO Event 40 was crossed with an oleic type non-GM safflower variety Montola 2003. Fatty acid analysis was undertaken on seed from the F2 generation. The number of F2 plants that contained levels of Oleic acid greater than 90% and Palmitic acid levels less that 3% were evaluated with respect to a 3:1 segregation ratio of the dominant SHOSO trait. Based on the analysis, the insertion of pCW732 in Event 40 is stably inherited and segregates in accordance with Mendelian inheritance.

Materials and methods

An individual plant of Event 40 (T4 generation) was grown in a glasshouse alongside a non-GM plant with a high oleic genotype (variety Montola 2003). During flowering, the two plants were manually crossed and all unused florets were emasculated to prevent self-pollination. This procedure generated forced cross seeds, termed F1 seed. The F1 seed were further grown to maturity and the flowers self-pollinated to generate a population of 59 F2 seed. Each of the 59 seed were analysed for their fatty acid profile, indicative of inheritance of the SHOSO trait.

Fatty acid analysis was undertaken on F2 safflower seeds. Firstly, the safflower seeds were imbibed on wet filter paper overnight (16 hrs) at room temperature to allow the seed coat to be easily removed. The tip of the cotyledons (5 mm) were excised and sampled for fatty acid methyl ester analysis (FAME). The remaining seed was planted in the glasshouse and grown to maturity.

FAMEs were prepared essentially as described by (Zhou et al., 2013), with slight modifications. The methylation was extended to 4 hr with scaled up to 800 µL 1N methanolic-HCl (Supelco, Bellefonte, USA). GC analysis with FID was also performed as described by (Zhou et al., 2013), except the ramping program was changed to an initial temperature at 150°C holding for 1 min, then raised to 180°C at 10°C/min, and to 240°C at 50°C/min holding for 4 min. GLC standard 411 (Nuchek, Elysain, USA) was used for calibration.

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

Results

Of the 59 F2 seed produced from the Event 40 x Montola 2003 cross, 14 individuals had Palmitic acid levels greater than 3%. Similarly, 14 individuals had Oleic acid levels equal to or less than 90%. These results are indicative of plants that do not contain a functional Event 40 insert. Chi-square goodness of fit tests indicated that there was no significant departure from the predicted 3:1 segregation ratio (Palmitic acid greater than 3% X^2[1,N=59] = 0.05, P=0.822, p<0.01; Oleic acid levels less than 90% X^2[1,N=59] = 0.277, P=0.0.599, p<0.01).

Crossing Event 40 with a linoleic type non-GM safflower

The SHOSO Event 40 was also crossed with a linoleic type non-GM safflower breeding line (low oleic/high linoleic). Fatty acid analysis was undertaken on seed from the F2 generation. The number of F2 plants that contained levels of Oleic acid greater than 90% and plants with linoleic levels greater than 70% were evaluated with respect to a 3:1 segregation ratio. Based on the analysis, the insertion of pCW732 in Event 40 is stably inherited and segregates in accordance with Mendelian inheritance.

Materials and methods

An individual plant of Event 40 (T4 generation) was grown in a glasshouse alongside a non-GM plant with a linoleic genotype (CSIRO safflower breeding line). During flowering, the two plants were manually crossed and all unused florets were emasculated to prevent self-pollination. This procedure generated 4 forced cross seeds, termed F1 seed. The F1 seed were further grown to maturity and the flowers self-pollinated to generate a population of 126 F2 seed. Each of the 126 seed were analysed for their fatty acid profile, indicative of inheritance of the SHOSO trait.

Fatty acid analysis was undertaken on F2 safflower seeds. Firstly, the safflower seeds were imbibed on wet filter paper overnight (16 hrs) at room temperature to allow the seed coat to be easily removed. The tip of the cotyledons (5 mm) were excised and sampled for fatty acid methyl ester analysis (FAME). The remaining seed was planted in the glasshouse and grown to maturity.

FAMEs were prepared as described above.

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

Results

The fatty acid composition of the F2 population was analysed. As expected from a cross between a Linoleic type and a High Oleic type, levels of Linoleic acid were negatively correlated with Oleic acid (Arslan 2007; Geçgel et al., 2007; Figure 19).

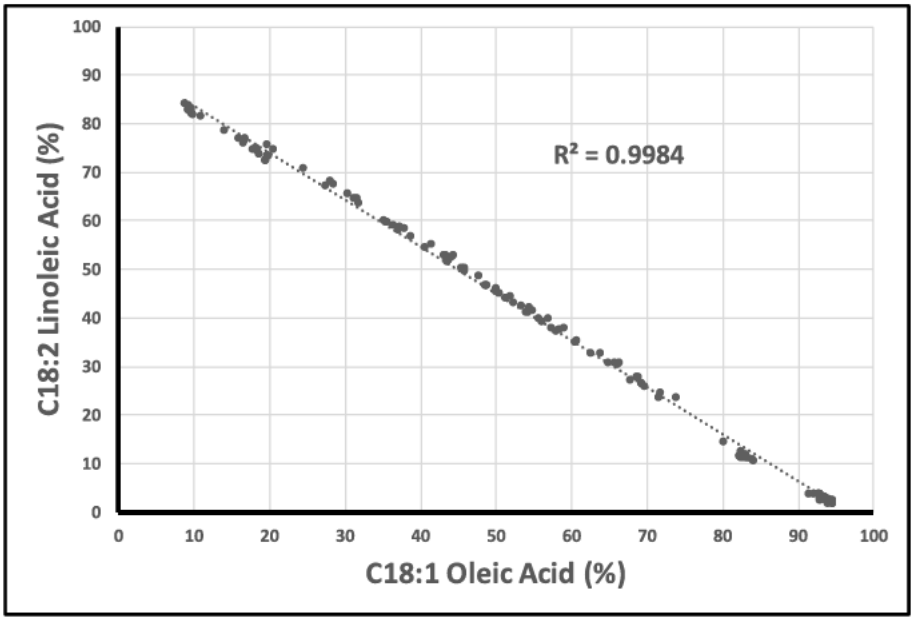


Figure 19. Negative correlation between linoleic and oleic acid in a cross between Event 40 and a linoleic breeding line.

A total of 126 F2 seeds from a cross between Event 40 and a linoleic type breeding line were analysed by FAME analysis. As expected, the percentage of Linoleic acid is negatively correlated with Oleic acid. As expected from a cross between a Linoleic type and a High Oleic type, levels of Linoleic acid were negatively correlated with Oleic acid (Arslan 2007; Geçgel et al., 2007).

Of the 126 F2 seed produced from the Event 40 x Linoleic safflower breeding line cross, 27 individuals had Oleic acid levels equal to or greater than 90%. These results are indicative of plants that contain a functional Event 40 insert (i.e. super high oleic). Chi-square goodness of fit tests

indicated that there was no significant departure from the predicted 3:1 segregation ratio (Oleic acid greater than 90% X^2[1,N=126] = 0.68, P=0.4096, p<0.01).

Of the 126 F2 seed, 24 individuals had linoleic acid levels greater than 70%. These results are indicative of plants that contain no functional Event 40 insert and are comparable to the linoleic safflower breeding line. Chi-square goodness of fit tests indicated that there was no significant departure from the predicted 3:1 segregation ratio (Linoleic acid greater than 70% X^2[1,N=126] = 2.08, P=0.1492, p<0.01).

Inheritance of the trait fatty acid profile

Event 26 and Event 40 safflower have been genetically modified to accumulate super high levels of oleic acid in the seed. The fatty acid composition of 7th generation seed from field grown safflower were analysed for their fatty acid profiles (Table 7). As expected, in Event 26 and Event 40, the polyunsaturated fatty acid linoleic acid and monounsaturated oleic acid and the saturated fatty acid palmitic acid were significantly different (p<0.01) to the parental control M1582 (Table 7). Other fatty acid components remained comparable to the parental M1582 control. Observation of the expected phenotype from 7th generation seed material demonstrates the stability of the down regulation of *CtFAD2* and *CtFATB*.

Table 7. Fatty acid profile of Event 26 and Event 40 compared to the parental control M1582

Fatty Acid Component*	Event 26**	Event 40**	M1582**
Palmitic acid C16:0	3.46±0.13[a]	3.53±0.24[a]	6.27±0.12[b]
Palmitoleic acid C16:1	0.07±0.04[a]	0.12±0.07[a]	0.03±0.01[a]
Stearic acid C18:0	1.33±0.12[a]	1.64±0.12[ab]	1.76±0.09[b]
Oleic acid C18:1	92.50±0.28[a]	91.82±0.49[a]	75.91±0.59[b]
Linoleic acid C18:2	2.52±0.22[a]	2.65±0.28[a]	15.73±0.58[b]
Linolenic acid C18:3	0[a]	0[a]	0[a]
Arachidic acid C20:0	0.14±0.03[a]	0.20±0.04[a]	0.23±0.03[a]

* Seed samples were analysed by NSW Department of Primary Industries Oil Testing Service and CSIRO. Fatty acid levels are presented as a percentage of total fatty acids (%). Values are means ± standard error. Means with the same letter are not significantly different (>0.01).
** Seed samples from the T7 generation were analysed from across 5 independent field trials conducted in 2016 under DIR131.

Summary of genetic stability studies

Crosses between each of the events to a non-GM safflower line demonstrated segregation in accordance with predicted inheritance ratios. Further, the lipidomic profiles of each event grown in the field over multiple generations and across various environments demonstrated the stability of the down regulation of the *CtFAD2* and *CtFATB* genes.

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

The pCW732 insert is fully functional in Event 26 and Event 40. The intended down regulation of *CtFAD2.2* and *CtFATB* are demonstrated by:

- Lipidomic analysis of advanced generation, field grown safflower showing reduced Palmitic acid (<3%), high Oleic acid (>90%) and reduced Linoleic acid (<2%)–see Table 7
- Profiling membrane-associated lipid species of different developmental stages from Event 26 and Event 40 compared with non-GM safflower varieties
- Reduced transcript abundance of both *CtFATB* and *CtFAD2.2* in Event 26 and Event 40
- sRNA mapping to *CtFAD2.2* and *CtFATB* demonstrating the creation of small RNA populations that lead to the down regulation of *CtFATB* and *CtFAD2.2.*

Further, Western blot analysis demonstrates that the hygromycin resistance gene is also functional in Event 26 and Event 40.

Profiling membrane-associated lipid species of different developmental stages

A comprehensive lipidomics analysis of seed and non-seed tissues in Event 26 and Event 40 was compared to high oleic and low oleic safflower varieties.

Materials and methods

Total lipids were extracted from freeze-dried cotyledon, hypocotyl, roots and true leaves of two-week-old safflower varieties. The varieties included: high oleic GM safflower Event 26 and Event 40 (SHO); high oleic non-GM safflower variety S-317 (HO1) and Lesaf496 (HO2); a high oleic non-GM safflower variety developed by ems mutagenesis (ems/S901) that has very high Oleic acid content in the seed (~90%), but compromised yield (Weisker, 1997); and low Oleic non-GM safflower variety Centennial (LO).

Freeze dried leaf tissue was ground to powder in a micro-centrifuge tube containing a metallic ball using Reicht tissue lyser (Qiagen) for 3 min at 20 frequency/s. Chloroform:methanol (2:1, v/v) was added and mixed for a further 3 min on the tissue lyser before the addition of 1:3 (v/v) of 0.1 M KCl. The sample was then mixed for a further 3 min before centrifugation (5 min at 14,000 g), after which the lower lipid phase was collected. The remaining phase was washed once with chloroform, and the lower phase extracted and pooled with the earlier extract. Lipid phase solvent was then evaporated completely using N_2 gas flow and the lipids re-suspended to 20 mg/ml chloroform per mg of the extracted oil.

Lipidomics analysis via LC-MS: Lipids extracts were diluted in 1:100 mL butanol:methanol (1:1, v/v) and analyzed by liquid chromatography-mass spectrometry (LC-MS), based on previously described methods (Reynolds et al., 2015). Briefly, an Agilent 1290 series LC and 6490 triple quadrupole LC-MS with Jet Stream ionisation. The phosphatidylcholine (PC) and lysophosphatidylcholine (LPC) species were separated on an Agilent 120 HILIC column (2.1 x 100 mm, 2.7 µm), over a gradient from 95% acetonitrile to 75% acetonitrile with 20 mM ammonium acetate. PC and LPC hydrogen adducts were quantified by the characteristic 184 *m/z* phosphatidyl head group ion under positive ionisation mode. The ammonium adducts of monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG), diacylglycerol (DAG) and TAG lipid species were analyzed by the neutral loss of singular fatty acids C_{16} to C_{20}. Multiple reaction monitoring (MRM) lists were based on the following major fatty acids: 16:0, 16:3, 18:0, 18:1, 18:2, 18:3, using a collision energy of 28 V. Lipids were chromatographically separated using an Agilent Poroshell column (50 mm x 2.1 mm, 2.7 µm) and a binary gradient with a flow rate of

0.2 mL/min. The mobile phases were: A. 10 mM ammonium formate in H_2O:acetonitrile: isopropanol (5:45:50, v/v); B. 10 mM ammonium formate in H_2O:acetonitrile: isopropanol (5:20:75, v/v). Individual MRM TAG was identified based on ammoniated precursor ion and product ion from neutral loss. Results were integrated using Agilent Mass Hunter Quantitative software and exported into R for statistical and graphical analysis.

Results for Event 26 and Event 40

Lipid profiling of various tissues across a range of non-GM and SHOSO Event 26 and Event 40 safflower indicate that the transgenic RNA interference approach is restricted to seed and developmentally-derived organs, such as the emergent cotyledons and hypocotyls (Figure 20 and Figure 21).

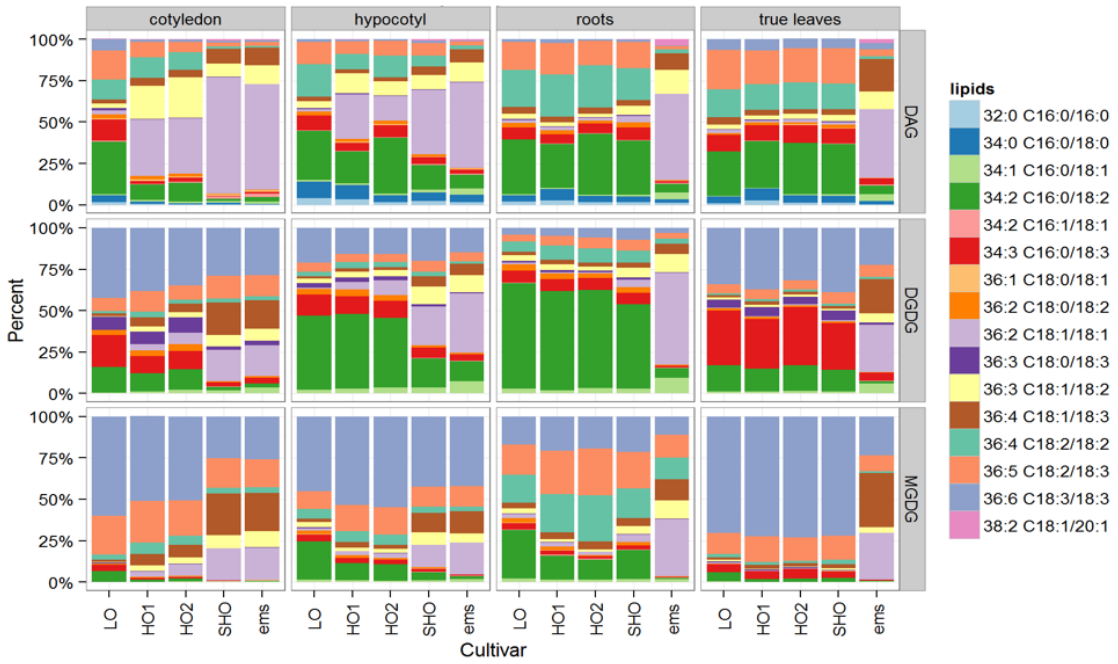


Figure 20. Profiling membrane-associated lipid species of different developmental stages from Event 26 and non-GM safflower varieties.

The analysis includes varieties that have altered seed oleic profiles, such as low oleic (LO; Centennial), high oleic (HO1, S317; HO2, Lesaf496), super high oleic (SHO, Event 26) and S901 (ems). Other SHO lines were analysed and display similar trends for Event 26. Diacylglycerol (DAG), digalactosyldiacylglycerol (DGDG) and monogalactosyldiacylglycerol (MGDG).

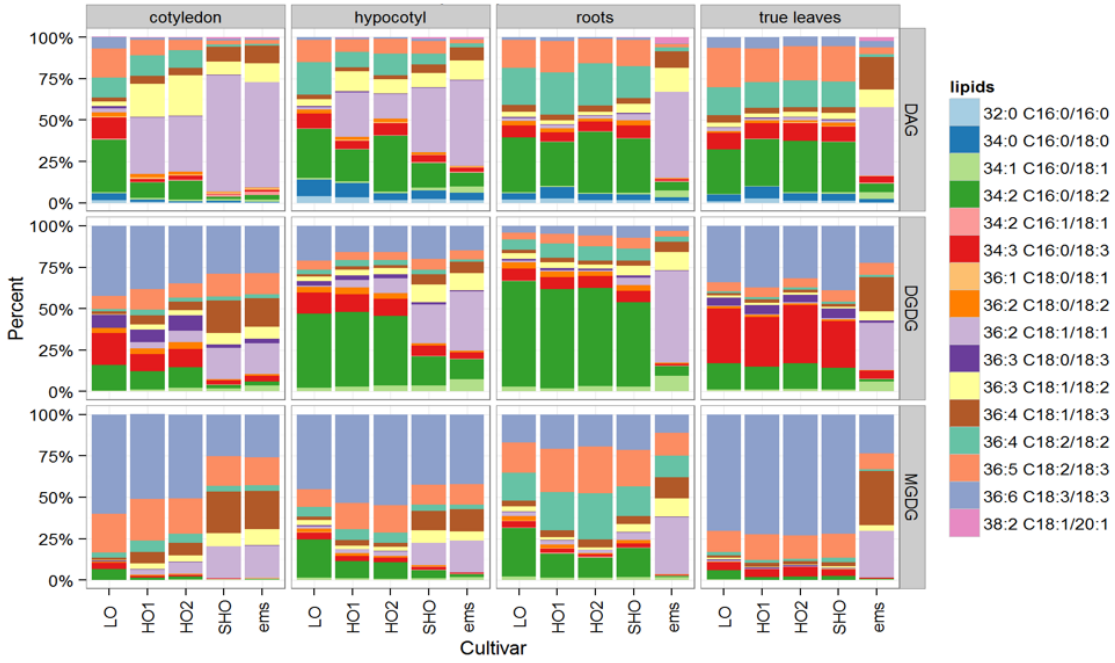


Figure 21. Profiling membrane-associated lipid species of different developmental stages from Event 40 and non-GM safflower varieties.

The analysis includes varieties that have altered seed oleic profiles, such as low oleic (LO; Centennial), high oleic (HO1, S317; HO2, Lesaf496), super high oleic (SHO, Event 40) and S901 (ems). Other SHO lines were analysed and display similar trends for Event 40. Diacylglycerol (DAG), digalactosyldiacylglycerol (DGDG) and monogalactosyldiacylglycerol (MGDG).

This analysis found that in roots and true leaves, the dominant lipid species such as diacylglycerol (DAG), digalactosyldiacylglyercol (DGDG) and monogalactosyldiacylglyercol (MGDG) were unchanged between LO, HO, Event 26 and Event 40 and showed the high polyunsaturated fatty acid composition typical of these vegetative tissues (Figure 20 and Figure 21). In contrast, the non-GM safflower (ems/S901) contained a marked increase in the monounsaturated content relative to the polyunsaturated fatty acids.

Down regulation of *CtFATB* and *CtFAD2.2* in Event 26 and Event 40

Quantitative PCR was used to assess the abundance of *CtFAD2.2* and *CtFATB* in SHOSO events relative to a non-GM high Oleic safflower variety S-317. This analysis found that the abundance of *CtFAD2.2* and *CtFATB* in Event 26 and Event 40 were significantly reduced compared to non-GM safflower expression levels.

Materials and methods

GM and non-GM safflower plants were grown in glasshouses until flowering. Florets were manually self-pollinated to set the time of seed development and developing embryos were sampled for total RNA 15 days after pollination.

Total RNA was extracted from maturing safflower seed as outlined above, however the precipitated RNA was further cleaned of small RNA using Plant RNAeasy columns (Qiagen). cDNA synthesis was carried out using Superscript III reverse transcriptase (Life Technologies, Invitrogen)

according to the manufacturers protocol with an oligo dT primer (Invitrogen). For each RNA sample, three separate cDNA synthesis reactions were carried out. Real-time quantitative (qRT)-PCR was carried out as described (Allen et al., 2007). Data analysis was performed using SPSS Statistics (version 23) with the significance of differences between means tested using the Least Significant Difference (LSD) test ($p<0.05$).

Results

The expression levels of *CtFATB* and *CtFAD2.2* are significantly reduced ($p<0.05$) in Event 26 and Event 40 compared to high Oleic non-GM safflower (Figure 22). For *CtFATB*, Event 26 and Event 40 were significantly different ($p<0.01$) from each other but not significantly different ($p>0.05$) in *CtFAD2.2* expression.

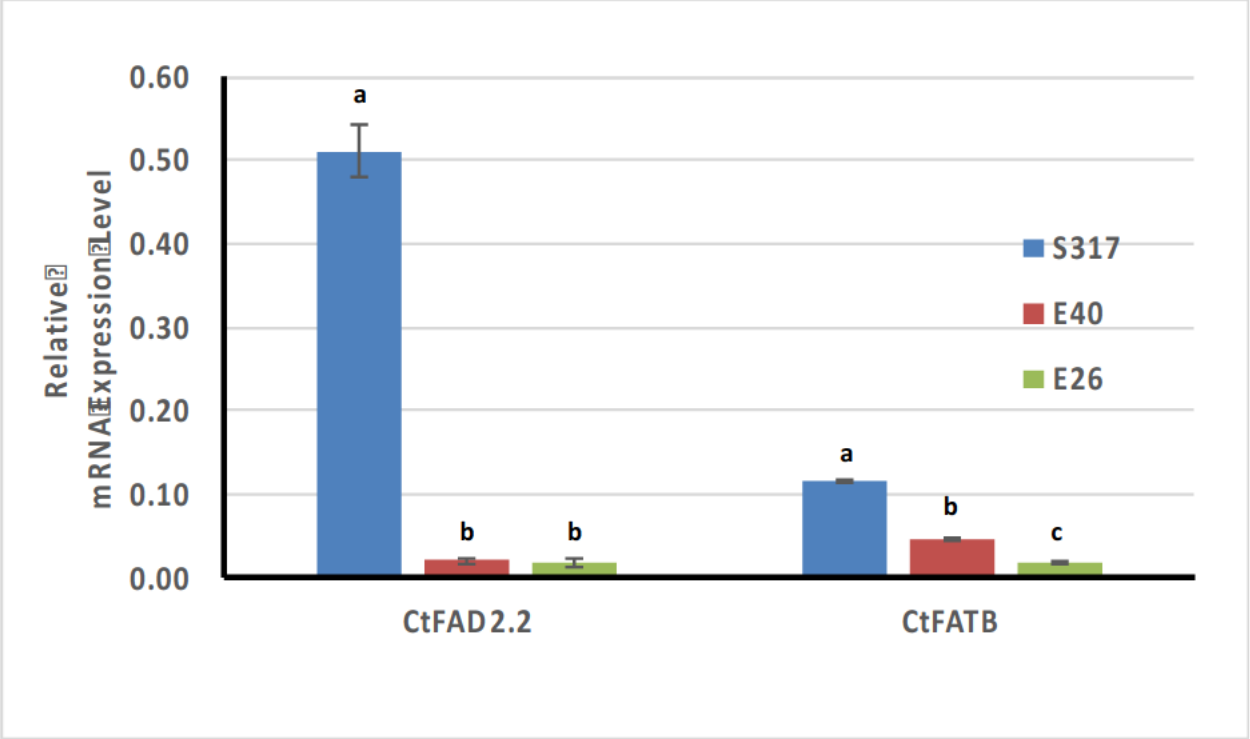


Figure 22. Down regulation of *CtFAD2.2* and *CtFATB* in Event 26 and Event 40.

An average of three biological replicates and three technical replicates were assessed for each event and non-GM safflower control. Event 26 and Event 40 have significantly reduced ($p<0.05$) levels of transcript abundance for *CtFAD2.2* and *CtFATB* compared to the non-GM safflower control. Mean relative mRNA expression levels with the same letter are not significantly different ($p>0.05$).

Small RNA mapping of *CtFAD2.2* and *CtFATB* in Event 26 and Event 40

Small RNA, generally in the size range 21-24 nt, extracted from Event 26 and Event 40 were deep sequenced and these sequences were mapped against the draft safflower genome (CSIRO in house data resource). The only locations where these small RNA populations map against were *CtFAD2.2* and *CtFATB* (Figure 23 and Figure 24). The locations of the alignments were confined to the areas that were used in the design of the hairpin vector pCW732. A wider examination of siRNA aligning elsewhere in the safflower genome failed to find any significant hits, defined here as threshold of 10 hits across an open reading frame (ORF).

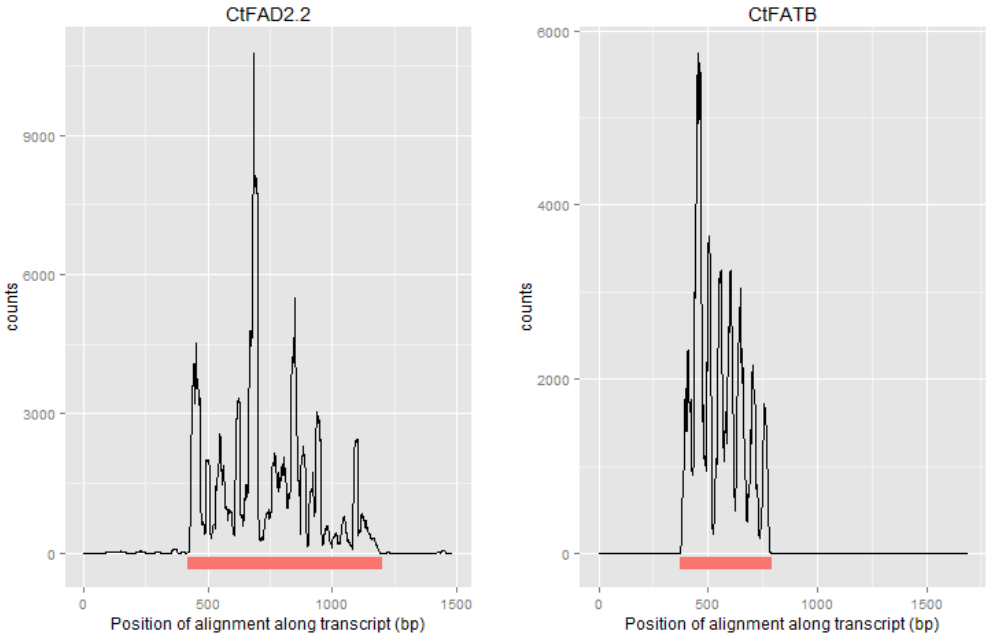


Figure 23. Populations of sRNA mapping to *CtFAD2.2* and *CtFATB* in Event 26.

From developing embryos, approximately 15 days after anthesis, small RNA was isolated from SHO Event 26 and subjected to deep sequencing. The small RNA populations were back aligned to the safflower genome and the T-DNA vector pCW732. The red bar in each plot represents the position of the RNAi fragment in pCW732 aligning with the transcript of the target gene, *CtFAD2.2* or *CtFATB*, respectively.

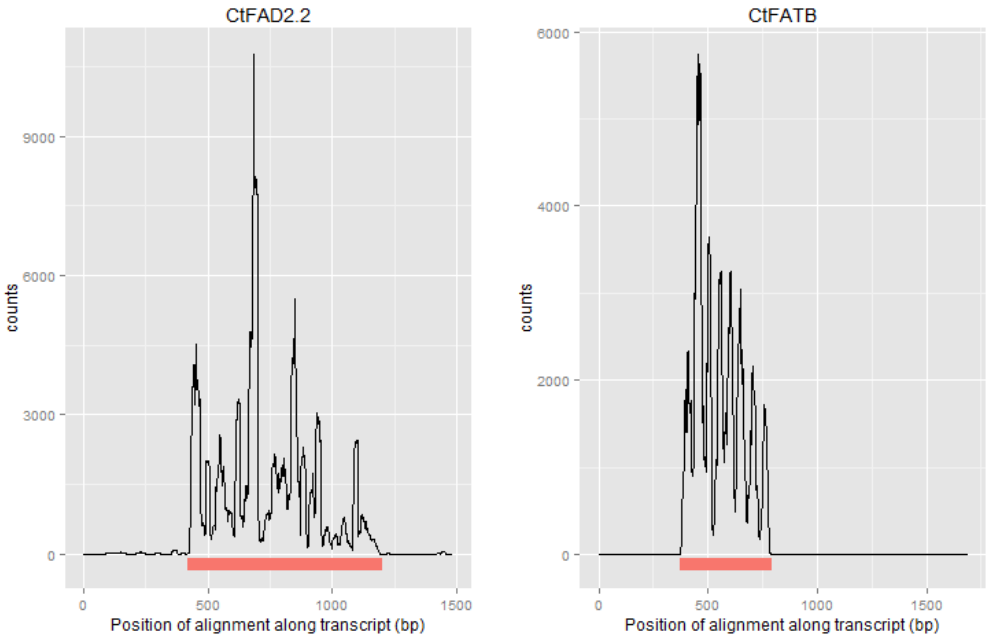


Figure 24. Populations of sRNA mapping to *CtFAD2.2* and *CtFATB* in Event 40.

From developing embryos, approximately 15 days after anthesis, small RNA were isolated from SHO Event 40 and subjected to deep sequencing. The small RNA populations were back aligned to the safflower genome and the T-DNA vector pCW732. The red bar in each plot represents the position of the RNAi fragment in pCW732 aligning with the transcript of the target gene, *CtFAD2.2* or *CtFATB*, respectively.

Conclusion of the genetic characterisation of Event 26 and Event 40

Molecular analyses demonstrated that Event 26 and Event 40 each contained a single insert from pCW732. Each insert from pCW732 integrated at a single locus.

A combination of Genome Walking and DNA sequence analysis was used to determine the structure of each insert in the events. The insertion of pCW732 T-DNA in Event 26 generated a 69 bp deletion within the genomic region and the entire Left Border sequence (160bp) was inserted into the genome, but only 41 bp of the162 bp Right Border sequence was inserted. Sequence analysis also revealed that 191 bp of the replication of origin RiA4 from the binary vector pORE-CBIb was also inserted. No backbone sequences were detected.

The insertion of pCW732 T-DNA in Event 40 generated a 34 bp deletion and 35 bp duplication within the genomic region and both the Left Border (16bp) and Right Border (39 bp) sequences were truncated. No backbone sequences were detected.

Crosses to non-GM safflower and lipid analysis studies confirmed the stability of the DNA inserts and seed specific down regulation of *CtFAD2* and *CtFATB* in Event 26 and Event 40 across multiple generations and environments.

B. Characterisation and Safety Assessment of New Substances

B.1. Characterisation and Safety Assessment of New Substances

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

Mode of action and phenotypic effects of Hygromycin phosphotransferase

Tissue culture methods for the generation of transgenic safflower require a selectable marker system to allow isolation of genetically modified materials and bias against non-GM safflower. In the vector pCW732, the Hygromycin resistance protein (HPH) is encoded by the *hph* gene expressed by the constitutive promoter 35S (Table 2 and Figure 3). The HPH protein is the only protein overexpressed from pCW732. All other elements provide down regulation of endogenous safflower fatty acid genes.

Details on the mode of action, phenotypic effects and safety of the HPH protein have been reviewed (Bennet et al., 2004; Peterson et al., 2005; OGTR 2012; Ramessar et al., 2007) and can be summarised as follows:

- The HPH protein catalyses the phosphorylation of the 4-hydroxyl group of the antibiotic Hygromycin B, rendering it inactive. This is highly specific for a limited number of antibiotics
- The *hph* gene has been used as a selectable marker since 1985 and has been approved in a large number of crops and field trials
- Several cotton products containing the *hph* gene have been assessed and approved by FSANZ (see A509 and A615)
- The antibiotic Hygromycin B is not used for human clinical applications and has no effect on aminocyclitol or aminoglycoside antibiotics
- In animal studies, the protein has no acute toxicity and database analysis reveals no similarity to known toxic proteins or allergens
- The protein is not glycosylated in plants and the protein is rapidly degraded in gastric fluid
- The US EPA (2004) established an exemption from the requirement of a tolerance for residues of the marker protein on cotton and the European Food Safety Authority (2004) have no restrictions of the use of the marker protein as a selectable marker for field testing or commercialisation.

Functional Hygromycin phosphotransferase in Event 26 and Event 40

Detection of HYH protein was achieved via the use of specific HYH binding antibodies.

Materials and methods

Seeds from field grown Event 26, Event 40 (T8 generation) and the non-GM safflower M1582 were sown to soil. Samples of true leaves (2 cm diameter) were harvested after 3 weeks growth, and subjected to western blot analysis as previously described (Belide et al., 2011). Briefly, leaf material was ground in liquid nitrogen to a powder, and approximately 5 mg of powder was added to 300 µL of standard Laemmli Buffer, heated to 95°C for 5 minutes, cooled to room temperature, centrifuged at 10000 rpm for 5 minutes and 20 µL of the supernatant applied to a denaturing SDS

gel (4-12% gradient; Life Technologies). Proteins were resolved at 200 mA for 40 minutes, using a BenchMark protein ladder as a standard for sizing. Proteins were blotted onto prepared PVDF membrane and probed using a primary mouse monoclonal antibody raised against HYH protein (HYHmb; mybioSource.com product MBS857772; 1:2000 dilution) and a secondary antibody (anti-mouse HRP; 1:5000). Positive controls to HYH (F11576 and F11772) were included on all membranes. F11772 was an F1 hybrid between Event 40 and a non-GM safflower, where all alleles are expected to be hemizygous. F11576 is an individual for another SHSO plant (Event 33), containing ~5 T-DNA copies.

Results

Analysis of Event 26 and Event 40 demonstrated an expected single band of 40 kD corresponding to the HYH protein (Figure 25 and Figure 26). There was no evidence of multiple protein products and no HYH was detected in the non-GM safflower M1582.

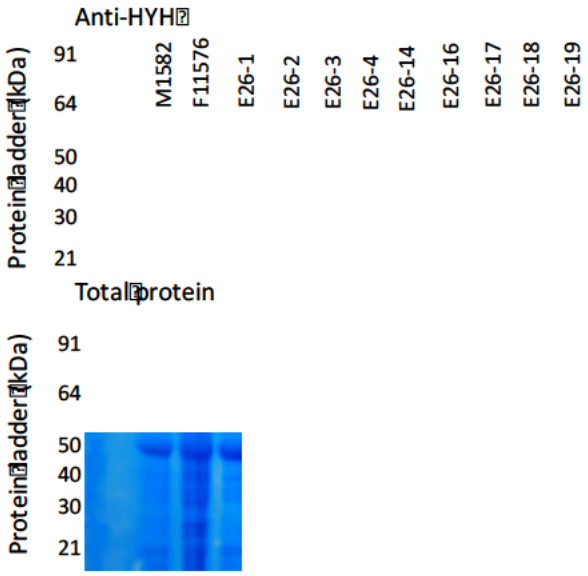


Figure 25. Western blot analysis of the HPH protein in transgenic safflower Event 26

Upper panel, western blot of total protein extracts of leaves; lower panel, protein loading of each sample. M1582: Non-GM safflower; F11772 and F11576 are internal controls for expression of HYH (HPH) protein; E26-1, E26-2, E26-3, E26-4, E26-14, E26-16, E26-17, E26-18, E26-19 are samples taken from different plants grown from seed harvested from a GM field trial undertaken in 2016.

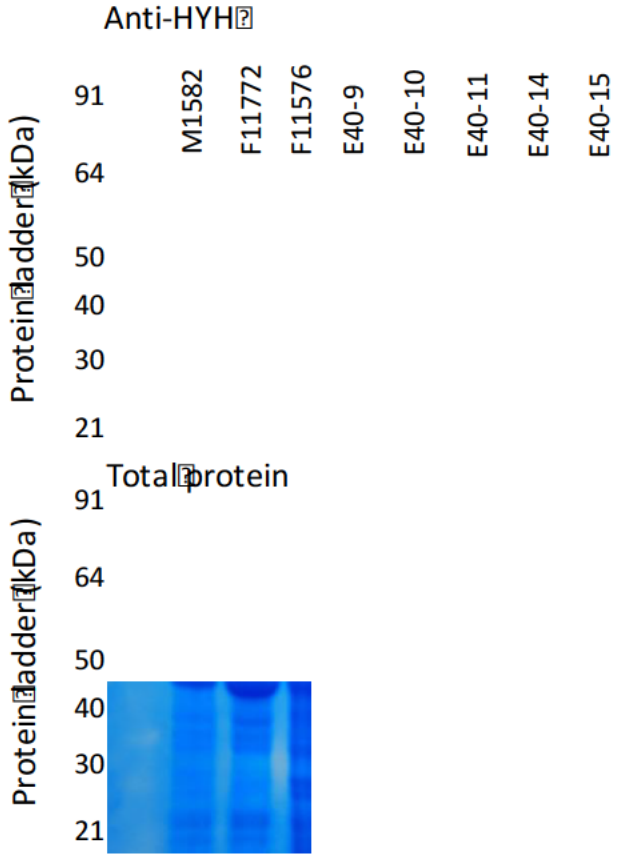


Figure 26. Western blot analysis of the HPH protein in transgenic safflower Event 40.

Upper panel, western blot of total protein extracts of leaves; lower panel, protein loading of each sample. M1582: Non-GM safflower; F11772 and F11576 are internal controls for expression of HYH (HPH) protein; E40-9, 10, 11, 14 and 15 are samples taken from different plants grown from seed harvested from a GM field trial undertaken in 2016.

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

Details on the mode of action, phenotypic effects and safety of the HPH protein have been reviewed (Bennet et al., 2004; Peterson et al., 2005; OGTR 2012; Ramessar et al., 2007) and are summarised above in Section B.1(a).

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

The HPH protein is expressed from a modified bacterial gene. The transformed safflower was selected in the laboratory in the presence of Hygromycin B. Post-translational modifications (PTMs) to HPH cannot be directly evaluated as expression levels are below the limit of detection. The protein is not naturally glycosylated in plants (Peterson et al 2005).

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

An analysis was completed to assess open reading frames (ORFs) in the Super High Oleic Acid Safflower Oil Event 26 and Event 40, which was generated by transformation of the high Oleic acid breeding line M1582 with pCW732 T-DNA. Novel ORFs were evaluated using bioinformatics (i.e., amino acid sequence comparison techniques) to assess their similarity to known toxins or allergens. Details of this analysis are provided in A.3(c)(v).

Bioinformatic analyses of 65 start-to-stop ORFs within the DNA insert and genomic junction regions of Event 26 and 62 start-to-stop ORFs within the DNA insert and genomic junction regions of Event 40, demonstrated no putative polypeptides with relevant homology to proteins that are known to be toxic, allergenic, or to have other biologically adverse properties.

B.2. New Proteins

B.2 (a) and (b) Information on potential toxicity and allergenicity

The pCW732 T-DNA contains the hygromycin gene. The gene is expressed under the constitutive Cauliflower mosaic virus promoter. A detailed description of the history and mode of action of HPH can be found in Section B.1(b).

Details of the potential toxicity and allergenicity of the protein HPH are presented in the following Sections:

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

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

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

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

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

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

Transcription of the inverted repeats leads to down regulation of *CtFAD2.2* and *CtFATB* through production of dsRNA and the plant's RNAi pathway. The inverted repeats are derived from the DNA sequences from two safflower genes (*CtFAD2.2* and *CtFATB*; Table 1).

The T-DNA in the plasmid pCW732 contains a down-regulation cassette that results in the production of siRNA in Event 26 and Event 40 using the plant's RNAi pathway. As described in Section A.3(b), each down-regulation cassette is comprised of DNA sequence arranged as an inverted repeat.

Due to the nature of the inverted repeat sequences, their transcripts form dsRNA through complementary binding. The dsRNA act as a precursor for the plant's own RNAi post-transcriptional regulatory pathway. A cellular RNase III enzyme, Dicer, recognises and processes the precursor dsRNA into 21-24 bp duplexes termed siRNA. The siRNA bind with cellular proteins forming RNA Induced Silencing Complexes (RISC). The RISC selectively degrades one of the siRNA strands, referred to as the passenger strand. The remaining strand, referred to as the guide strand is used to target the complementary sequence in mRNA molecules. Once the guide strand pairs with an mRNA in a RISC complex, the mRNA molecule is cleaved and degraded, preventing protein translation (Chau and Lee, 2007; Fire et al., 1998).

Safety Assessment of Small RNA Generated in Event 26 and Event 40 Safflower

Increased oleic acid content in safflower seed was achieved using RNAi to down regulate *CtFAD2* and *CtFATB* in Event 26 and Event 40. This resulted in reduced levels of mRNA transcripts for these enzymes in the seed.

A weight-of-the-evidence approach was used to support the safe consumption of small RNA in Event 26 and Event 40 safflower. The analysis included:

- The history of safe use of dsRNA and siRNA in food and feed
- A summary of the many biological barriers that limit the uptake and activity of small RNA in mammalian cells

- A bioinformatic analysis comparing complementarity of potential siRNAs from Event 26 and Event 40 inserts to transcripts in humans and livestock commonly fed safflower; and

History of safe use of dsRNA and siRNA

RNAi refers to a cellular pathway used by plant and animal cells to down regulate gene expression through degradation of targeted mRNA within the cell. Cellular enzymes detect long dsRNA and process them into small (21-24 nucleotide) interfering RNA (siRNA) (Hammond, 2005). The resulting siRNA pair with cellular proteins which use one strand of the siRNA sequence to bind complementary mRNA sequences. Once bound by the siRNA-protein complex, the targeted mRNA sequence is cleaved, preventing protein translation (Chau and Lee, 2007; Fire et al., 1998).

The use of siRNAs to regulate gene expression through processing of endogenous dsRNA is common and highly conserved in plants, insects, fungi, nematodes, and animals. Traditional breeding practices have resulted in a number of conventional cultivars that produce siRNA and dsRNA that down regulate genes (Parrott et al., 2010; Petrick et al., 2013). Soybean seed colour, maize stalk colour, and rice protein content (Della-Vedova et al., 2005; Kusaba et al., 2003; Tuteja et al., 2004) are all examples of traits obtained through conventional breeding that utilise endogenous siRNA and dsRNA to regulate gene expression.

Published scientific literature demonstrates that consumption of biotechnology-derived crops using RNA-based gene regulation, such as siRNA, microRNA (miRNA), or dsRNA, is as safe for humans and livestock as other crops using RNAi technology (Parrott et al., 2010; Petrick et al., 2013). Since 1994, over thirty RNAi based biotech events have been approved globally for food, feed or cultivation in crops including alfalfa, apple, bean, potato, squash, plum, papaya, soybean, and tomato. Together, these events make up over 130 food and feed approvals in sixteen countries and have been consumed safely by humans and animals (ISAAA, 2015).

RNA transcripts, such as dsRNA and siRNA, are composed of nucleotides that are the basic units of both DNA and RNA. They are regularly consumed as part of the diet and are considered safe:

- In 2001, the United States EPA established an exemption from the requirement for a tolerance for residues of nucleic acids (40 C.F.R. 174.507) under the Federal Food, Drug, and Cosmetic Act (FFDCA), noting that “nucleic acids are ubiquitous in all forms of life, have always been present in human and domestic animal food and are not known to cause any adverse health effects when consumed as part of food” (66 Fed. Reg. 37817, July 19, 2001)
- The United States FDA reached a similar conclusion, stating that nucleic acids are “generally recognised as safe” (57 Fed Reg. 22984, 22990, May 29, 1992). “Introduced nucleic acids, in and of themselves, do not raise safety concerns. Thus, for example, the introduction of a gene encoding an anti-sense ribonucleic acid (RNA) would not raise concerns about either the gene or the anti-sense RNA. Any safety considerations would focus on the intended effects of the anti-sense RNA”; and
- In 2013, the bi-national government agency, Food Standards Australia New Zealand, which evaluates food safety requirements from biotech foods stated, “There is no scientific basis for suggesting that small dsRNA present in some biotech foods have different properties or pose a greater risk than those already naturally abundant in conventional foods” (FSANZ, 2013).

In alignment with the history of international approvals, there is regulatory consensus on the history of safe consumption of RNA including RNA transcripts, such as dsRNA and siRNA.

Plant tissues have several mechanisms to produce long dsRNA. Analysis of plant transcriptomes suggests that dsRNA are abundant in plants, with over 8 million long dsRNA predicted in

conventional corn, soy, rice, lettuce, and tomato crops (Jensen et al., 2013). Many of these naturally abundant dsRNA have perfect complementarity to human genes and transcripts (Jensen et al., 2013). For example, a large number of small RNA in conventional rice grains have 100% complementary to nucleic acid sequences in the genomes and transcriptomes of mammals, including humans (Ivashuta et al., 2009).

The abundance of small RNA in food and their complementarity to animal genomes and transcriptomes provides strong evidence for a history of safe consumption, which is confirmed by the safe use of RNAi in approved biotechnology-derived crops.

Biological barriers to siRNA uptake

Many studies have examined the stability of small RNA and the biological barriers that limit uptake and activity of small RNA in mammals. Much of this research was aimed at optimising RNA for increased stability and improved uptake so that it could be used in therapeutics. RNA is extremely labile. Unlike DNA, RNA contains ribose with a hydroxyl group attached to the pentose ring in the 2' position. This hydroxyl group makes RNA less stable than DNA because it is more prone to hydrolysis. In addition, RNA is very susceptible to oxidation, hydrolytic cleavage by metallic complexes, and degradation by RNase enzymes (Fabre et al., 2014).

For humans and livestock that may eat safflower oil or meal, processing treatments such as changes in temperature and physical shearing associated with processing and preparation degrades RNA leading to loss of activity and shut down of cell metabolism that halts the replenishment of RNA. Oil is purified and would therefore not contain any protein or nucleic acid.

Exposure to siRNA is also reduced by numerous biological barriers that limit RNA uptake and activity in mammalian cells. A summary of biological barriers to RNA uptake from food and examples of associated studies include:

- Degradation in saliva during chewing
    - 62% loss of activity was observed after exposure to saliva for 10 minutes (Hickerson et al., 2008)
- Degradation in the gastro-intestinal (GI) tract and stomach
    - Approximately 95% of plant-derived miRNA in the GI tract and stomach were eliminated within 2 hours and this increased to 99.6% after 24 hours (Liang et al., 2014)
    - The level of maternal milk-derived miRNA found in the intestines of sucklings is three orders of magnitude lower than in the stomach following suckling (Title et al., 2015)
- Degradation in the rumen
    - Free RNA fed to sheep and cows was rapidly degraded in the rumen to oligonucleotides, nucleosides and bases (McAllan, 1982)
- Inhibition of absorption from the GI tract to the plasma
    - The charged polyanionic structure and relatively large size of siRNA restrict absorption (Witwer and Hirschi, 2014)
    - Absorption of plant miRNA delivered by feeding was measured in human plasma and was detectable over a range from 0 - 1.31% (Liang et al., 2015). Similar numbers were measured in mice (0.3-1.8%) when administered by oral gavage, which does not include salivary-based degradation (Liang et al., 2014)

  - Low levels in plasma were measured following oral gavage (0.3%) or direct intestinal injections (0.9-2.4%) of synthetic, non-hydrolysable, oligonucleotides (Nicklin et al., 1998; Raoof et al., 2004)
  - Small therapeutic oligonucleotides formulated to optimise stability and absorption have only managed to modestly increase this number to 9.5% (Tillman et al., 2008)
  - There was no evidence for uptake of maternal milk-derived miRNA in suckling intestinal epithelium, gastric epithelium, plasma, liver, or spleen cells (Title et al., 2015).

- Degradation in the plasma
  - siRNA duplexes incubated in mouse plasma were rapidly degraded with 100% degradation recorded within 10 minutes. The siRNA were shown to be hydrolysed to nucleosides in this process (Christensen et al., 2013)
  - A time course analysis was performed on the small number of plant-derived miRNA that were detectable in the plasma after administration to mice through ingestion. Most of the levels peaked at 6 hours and were undetectable within 9 hours of ingestion (Liang et al., 2014)
  - RNA molecules injected intravenously in mouse studies are rapidly cleared from the plasma by renal filtration and excretion (Petrick et al., 2013).
- Prevention of uptake from plasma into animal cells
  - The charged polyanionic structure and relatively large size of siRNA restrict absorption (Witwer and Hirschi, 2014) so that even tissue culture uptake requires the use of enhancing agents (e.g., lipofectamine)
  - Challenges associated with inefficient cellular uptake have hindered the use of small RNA as therapeutic agents (Singh et al., 2011).
- Cellular barriers to biological activity
  - Biological activity in the cell requires that small RNA escape endosomes to prevent their degradation (Pei et al., 2010)
  - Small RNA must be loaded onto a limited number of Argonaut proteins in the cell in order to serve as a guide strand for either miRNA or siRNA-based gene regulation (Wang et al., 2010)
  - Studies performed to evaluate the concentration requirements for small RNA have shown that 370 to 18,000 copies/cell of a small RNA were required to silence a target RNA by 50% to 87% when using non-hydrolysable small RNA and liposome-mediated transfection (Pei et al., 2010). This level of absorbed siRNA is unlikely based on the barriers to uptake and the absence of an siRNA amplification mechanism in mammalian cells; and
- Scalable impact on mammalian cells
  - Amplification of siRNA in the host cell typically requires RNA-dependent RNA polymerase which is not found in vertebrates (Petrick et al., 2013; Witwer and Hirschi, 2014). Thus, humans or livestock would need to consume siRNA continuously and have an efficient uptake mechanism to maintain siRNA at levels that effectively down regulate genes in mammalian cells.

Collectively, these data indicate that small RNA derived from Event 26 and Event 40 safflower will undergo degradation and encounter barriers that limit them from accumulating in mammalian cells at levels sufficient to affect gene expression. Generally, in order to affect an individual organism,

complete complementarity between siRNA and an off-target mRNA must exist. Furthermore, many cells in one or more organs would need to be adversely affected. Biologically significant levels of plant siRNA have not been detected in mammals consuming plant material.

Bioinformatic analysis of pCW732 derived siRNA

Down regulation through RNAi requires complementarity between siRNA and mRNA in targeted cells (Jensen et al., 2013). To determine if sequence complementarity exists between siRNA in Event 26, Event 40 and mRNA in humans, all the potential siRNA were queried against transcripts from NCBI's annotated RNA database (Pruitt et al., 2012).

RNA database searches

The NCBI database houses sequences for many organisms, but it can be queried (according to a taxonomy identifier) to target the search against specific organisms. A query using safflower transcripts provided a positive control for the searches.

Small RNA, extracted from Event 26 and Event 40 were deep sequenced and these sequences were mapped against the draft safflower genome (CSIRO in house data resource). The only locations where these small RNA populations map against were *CtFAD2.2* and *CtFATB* (Figure 23 and Figure 24). The locations of the alignments were confined to the areas that were used in the design of the hairpin vector pCW732. A wider examination of siRNA aligning elsewhere in the safflower genome failed to find any significant hits, defined here as threshold of 10 hits across an open reading frame (ORF).

Humans, cows, pigs, and sheep were selected for further analysis as they may have access to safflower oil and/or meal as a feed source.

In plants, dsRNA can be processed into 21 to 24-nt siRNA. The focus of this bioinformatic analysis was on the smallest species, 21-nt siRNA, as this will identify the most potential off-targets. The list of all possible siRNA sequences was created using a sliding-window algorithm that scanned the length of each inverted repeat stem (*CtFAD2* and *CtFATB*) to identify all possible contiguous 21-mers as independent entries. The algorithm output resulted in (n-20) independent siRNA sequences, where n is the length of the particular dsRNA region. Therefore, for *CtFAD2* there were 737 independent 21-nt sequences and for *CtFATB* there were 392 independent 21-nt sequences.

The refseq_rna database is the most comprehensive database of annotated RNA sequences available through NCBI and was used to search against mRNA transcripts. The current version of the database (Posted 3rd December 2017, 1:02 AM) contains 21,037,955 entries and 49,724,980,154 total letters. The query files contained all possible 21-nt sequences that could be derived from the double-stranded stem region of each inverted repeat (i.e. *CtFAD2* and *CtFATB*). A word-size of 21 identifies matches with complete complementarity to the potential siRNA. The BLASTn output format parameter defined the format of the output in a table. The entrez_query parameter was used to query the refseq_rna database for RNA associated with a particular class of organisms (i.e. human, cow, pig, sheep) based upon taxonomy identifier.

No human or livestock transcripts were identified with perfect complementarity to any of the 21-nt siRNA examined.

Conclusion of RNAi safety

According to scientific literature, RNA and siRNA are labile during processing and digestion and biological barriers further reduce potential exposure by limiting uptake of siRNA into the cells of mammals (Fabre et al., 2014; Hickerson et al., 2008; McAllan, 1982). There is no mechanism for

harm in consuming siRNA in Event 26 and Event 40 safflower due to RNA lability during processing and digestion, extensive biological barriers that limit uptake and activity in cells, and the lack of complementarity between the potential siRNA in Event 26 and Event 40 and the transcriptome and genome of humans and livestock.

Given that small RNA are ubiquitous in nature, present in all food, and unlikely to accumulate in the environment, consumption of Event 26 and Event 40 safflower and their associated siRNA is as safe as the consumption of conventional safflower.

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

The genetic and biochemical basis of lipid and oil biogenesis in safflower seed is well understood (Cao et al., 2012; Guan et al., 2012; Jones et al., 1995; Knutzon et al., 1992; Stymne and Appelqvist, 1978; Figure 27). The flux of lipid synthesis towards oleic acid can be prematurely diverted via the activity of FATB, a thioesterase transporting 16:0 from the chloroplast to the cytoplasm where this saturated fatty acid is available to accumulate into seed oil. Oleic acid produced in the chloroplast is exported to the cytoplasm via another class of thioesterases, FATA, and is made available for desaturation to linoleic acid, C18:2, via fatty acid desaturases (FAD2 enzymes) operating on phosphatidylcholine (PC) lipids in the endoplasmic reticulum.

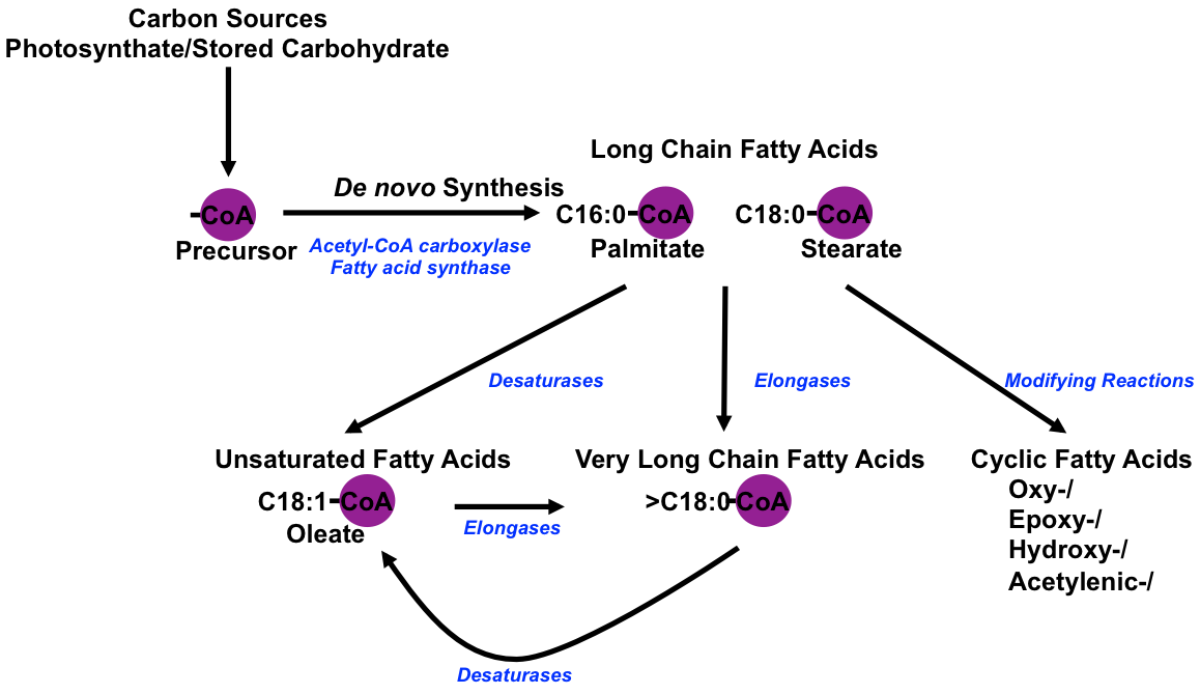


Figure 27. Fatty acid biosynthesis in plants (after Harwood 2005).

Safflower contains an extended family of it least 11 different FAD2 gene members (Cao et al., 2012), although only a *CtFAD2.1* and *CtFAD2.2* are oleic acid desaturases that are expressed in development of the oilseed. Subsequent studies have found that the *CtFAD2.1* is responsible for the majority of linoleic formation in seeds, via the *CtFAD2.1* mediated desaturation of oleic acid to linoleic acid in seed development (Liu et al., 2016). The identities of FATB in safflower remain to be discovered, although there are many similar sequences available in the literature to guide their identification. Nevertheless, a key question remains as to the enzymes responsible for the

remaining linoleic production in high oleic acid safflower and the FATB responsible for export of palmitic acid from the plastid in high oleic acid safflower.

With this context of the biogenesis of the fatty acids in safflower oilseeds, it has been previously demonstrated that reducing expression of FAD2 in seeds increases the oleic acid level. Furthermore, reducing expression of FATB in seeds decreases the palmitic acid level and reduction of both FAD2 and FATB activities simultaneously has been shown to increase the flux of lipids towards oleic acid more than down regulating FAD2 in isolation. Therefore, the key genes for the development of SHOSO in safflower is the correct identification of the seed active FAD2 and FATB genes, and subsequently the design of a strategy to co-suppress both genes via genetic modification.

A range of molecular biology investigations were conducted to identify safflower genes associated with the production of linoleic acid and palmitic acid.

To identify putative candidates with respect to residual linoleic fatty acid production and palmitic acid synthesis in safflower seed the expression of fatty acid genes was investigated by a deep sequence analysis of RNA extracted from developing embryos of low oleic acid (LOA) safflower and high oleic acid (HOA) safflower varieties. This analysis confirmed that *CtFAD2.1* transcript was reduced in HOA safflower relative to LOA safflower, however *CtFAD2.2* transcript abundance remained unchanged (Figure 28).

This approach also found a single transcript with homology to the family of saturated palmitic acid thioesterases, herein named *CtFATB*, and the abundance of this transcript was unchanged between HOA and LOA safflower. These results suggest that *CtFAD2.2* is the FAD2 desaturase that is responsible for the residual linoleic acid production in HOA safflower. This result also indicated that *CtFATB* is the thioesterase responsible for the synthesis of palmitic acid in developing seeds of safflower.

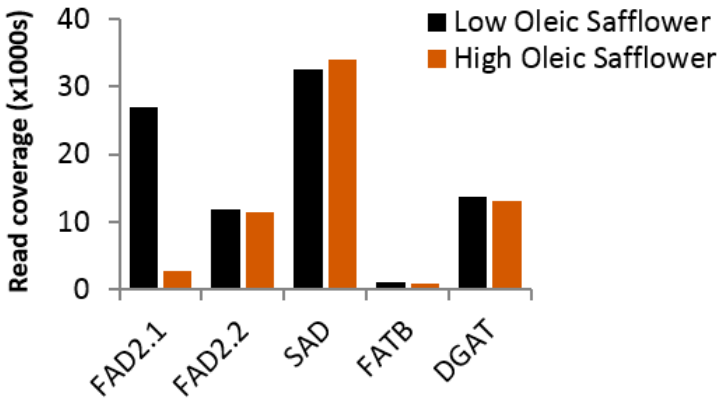


Figure 28. Deep sequencing analysis for the identification of the key genes associated with the residual linoleic and palmitic acids in high oleic safflower seed.

The graphic illustrates the expression profile of five selected genes in the developing embryos of safflower varieties, either low oleic acid safflower (LOA) with 80% linoleic acid, and high oleic acid safflower (HOA) with 80% oleic acid. Stearic acid desaturase (SAD) and diacylglycerolacyltransferase (DGAT) are included as representative genes that are not altered in their expression levels.

Collectively, the results indicate that down regulating the activities of *CtFAD2.2* and *CtFATB* in a HOA safflower background could reduce production of linoleic and palmitic acids and increase the flux of lipids into oleic acid during the development of safflower seeds.

This hypothesis was confirmed through the development and characterisation of safflower transformed with a cassette designed to down regulate *CtFAD2.2* and *CtFATB*. The fatty acid profile of Event 26 and Event 40 demonstrate a proportional increase in oleic acid (C18:1) percentage and proportional decreases in linoleic acid (C18:2) and palmitic acid (C16:0) compared to the parental line M5128 and other commercial check safflower varieties (see Table 7).

The growing scientific understanding and community recognition of the impact of the individual fatty acid components of food oils have on various aspects of human health is motivating the development of modified vegetable oils that have improved nutritional value while retaining the required functionality for various food applications. These modifications require knowledge about the metabolic pathways for plant fatty acid synthesis and the genes encoding the enzymes for these pathways (Liu et al., 2002; Thelen and Ohlrogge, 2002).

Considerable attention is being given to the nutritional impact of various fats and oils, in particular the influence of the constituents of fats and oils on cardiovascular disease, cancer and various inflammatory conditions. High levels of cholesterol and saturated fatty acids in the diet are thought to increase the risk of heart disease and this has led to nutritional advice to reduce the consumption of cholesterol-rich saturated animal fats in favour of cholesterol-free unsaturated plant oils (Liu et al., 2002).

While dietary intake of cholesterol present in animal fats can significantly increase the levels of total cholesterol in the blood, it has also been found that the fatty acids that comprise the fats and oils can themselves have significant effects on blood serum cholesterol levels. Of particular interest is the effect of dietary fatty acids on the undesirable low-density lipoprotein (LDL) and desirable high-density lipoprotein (HDL) forms of cholesterol in the blood. In general, saturated fatty acids, particularly myristic acid (C14:0) and palmitic acid (C16:0; Figure 27) have the undesirable property of raising serum LDL-cholesterol levels and consequently increasing the risk of cardiovascular disease (Zock, 1994; Hu et al., 1997). However, it has become well established that stearic acid (C18:0), the other main saturate present in plant oils, does not raise LDL-cholesterol, and may actually lower total cholesterol (Bonanome and Grundy, 1988; Dougherty, 1995). Stearic acid is therefore generally considered to be at least neutral with respect to risk of cardiovascular disease (Tholstrup, et al., 1994). On the other hand, unsaturated fatty acids, such as the monounsaturated oleic acid (also known as *cis*-Δ9-octadecenoic acid, (9*Z*)-octadec-9-enoic acid and C18:1; Figure 27), have the beneficial property of lowering LDL-cholesterol (Mensink and Katan, 1990; Roche and Gibney, 2000), thus reducing the risk of cardiovascular disease.

Oil processors and food manufacturers have traditionally relied on hydrogenation to reduce the level of unsaturated fatty acids in oils, thereby increasing their oxidative stability in frying applications and also providing solid fats for use in margarine and shortenings. Hydrogenation is a chemical process that reduces the degree of unsaturation of oils by converting carbon double bonds into carbon single bonds. Complete hydrogenation produces a fully saturated fat. However, the process of partial hydrogenation results in increased levels of both saturated fatty acids and monounsaturated fatty acids. Some of the monounsaturates formed during partial hydrogenation are in the *trans* isomer form (such as elaidic acid, a *trans* isomer of oleic acid) rather than the naturally occurring *cis* isomer.

In contrast to *cis*-unsaturated fatty acids, *trans*-fatty acids are now known to be as potent as palmitic acid in raising serum LDL cholesterol levels (Mensink and Katan, 1990; Noakes and Clifton, 1998) and lowering serum HDL cholesterol (Zock and Katan, 1992), and thus contribute to increased risk of cardiovascular disease (Ascherio and Willett, 1997). As a result of increased awareness of the anti-nutritional effects of *trans*-fatty acids, there is now a growing trend away from the use of hydrogenated oils in the food industry, in favour of fats and oils that are both

nutritionally beneficial and can provide the required functionality without hydrogenation, in particular those that are rich in either oleic acid where liquid oils are required or stearic acid where a solid or semi-solid fat is preferred.

Oil high in oleic acid also has many industrial uses (see review by Jaworski and Cahoon, 2003) This includes, but is not limited to, use in lubricant esters, biofuels, animal feeds, raw materials for fatty alcohols, plasticisers, waxes, metal stearates, emulsifiers, personal care products, soaps and detergents, surfactants, pharmaceuticals, metal working additives, raw material for fabric softeners, inks, transparent soaps, PVC stabiliser, alkyd resins, and intermediates for many other types of downstream oleochemical derivatives.

Safflower seeds produce oils that predominantly contain monounsaturated fatty acid (C18:1; oleic acid) and polyunsaturated fatty acid (C18:2; linoleic acid). While both have commercial uses, it is the valuable oleic acid that is used as a replacement to petroleum-based feed stocks in the manufacture of plastics, lubricants and cosmetics etc. Traditional breeding programs have developed safflower seed with oleic acid levels in the range of 75–85%, and are the highest purity sources of oleic acid in any oilseed. However, like other oilseeds, the remaining linoleic acid component, at 12–18%, is not desirable for industrial use because it is unstable and difficult to remove during oil processing.

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

The expression levels of transcripts from *CtFAD2* and *CtFATB* are presented in Section A.3(g).

The reduced expression was consistent with the compositional and trait efficacy data in Sections B.1 and B.5.

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

The reduced expression of the *CtFAD2* and *CtFATB* genes is facilitated by the seed specific Linin promotor from flax. The promoter is primarily active in seeds. The specificity of reduced expression is demonstrated in Section A.3(g).

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

This must include all of the following:

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

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

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

The nutritional composition and trait efficacy of safflower Event 26 and Event 40 was compared with its parental control, M1582. Commercially available reference varieties with a history of safe use for food and feed were, where applicable, included as comparators. Composite samples from geographically diverse field trials were assessed for those analytes important to safflower nutrition as well as those related specifically to trait efficacy. The nutritional assessment evaluating proximates, vitamins, minerals, amino acids, and anti-nutrients demonstrated that both Event 26 and Event 40 are compositionally equivalent to the parental control. As expected, the efficacy assessment evaluating the fatty acid profile demonstrated that both Event 26 and Event 40 have lower levels of Palmitic acid (C16:0) and Linoleic acid (C18:2), and higher levels of Oleic acid (C18:1) compared to M1582.

Several safflower products can also be used for animal feeds; seed meal–a by-product from the crushing process to obtain super high oleic oil and on rare occasions early to late stage vegetative tissues–for stock grazing. Analysis of seed meal and vegetative tissue demonstrates that both Event 26 and Event 40 could be considered not toxic and suitable for animal feed.

All methods were undertaken by commercial testing laboratories utilising technical procedures and methods in accordance with industry standards.

Materials and methods

Selection of control and reference varieties

Safflower seed was chosen as the primary test material for compositional analysis of Event 26 and Event 40 because meal and oil fractions are derived from seed. Compositional evaluation of seed would be representative of these derived materials. Composition analysis of vegetative tissue from field grown Event 26 and Event 40 was also examined.

The most relevant comparator for Event 26 and Event 40 is the advanced breeding line M1582, the parental variety. The only difference between Event 26 and Event 40 and the parental control is that Event 26 and Event 40 underwent transformation and contain pCW732 inserts.

Conventional non-transformed safflower varieties with a history of safe use for food and feed were also used as reference varieties. Both oleic type and linoleic type safflower varieties were included. These varieties are commonly used in bird seed and vegetable oil markets. The following reference varieties were included to provide a range of values common to conventional non-transformed safflower: Sironaria (Linoleic type), S-317, Montola 2003 and S901 (Oleic types). Further

comparisons included data obtained from papers published that examined safflower composition and nutrition from a range of safflower varieties and breeding lines.

The by-products, safflower meal, and early stage vegetative tissue may be used for animal feeding. As such, feed quality assessments were undertaken on Event 26 and Event 40 seed meal (derived from an expeller press) and field grown vegetative tissue.

Field Trials

Field trials were conducted for the purpose of phenotypic and agronomic assessment and to provide seed and vegetative tissue samples for molecular characterisation and compositional analysis (Table 8). Event 26, Event 40, their parental control (M1582) and conventional non-transformed safflower were grown across safflower growing regions in Australia under licences issued by the Office of the Gene Technology Regulator (DIR121 and DIR131).

The agronomic practices and pest control measures used were location-specific and were typical for all aspects of safflower cultivation and included soil preparation, fertilizer application, irrigation, and pesticide application.

The field trials were established in a randomised complete block (RCB) design. The treatments included the test, control, and reference varieties. Every block (replicate) included a plot of each treatment. The experimental unit was the plot. All plots within each block were independently randomised so that the treatments were in random order.

Typically, there were four replicates at each site. Within each replicate, each safflower variety was planted in plots arranged in random order.

Although some trials occurred in the same State, they were not planted in the same location. Plots were in different fields, or in different locations on the farm due to crop rotation practices. Field conditions such as environment, field history, soil type, pest presence, and drainage can differ from year to year. Each State was considered a unique site.

At least two geographically distant sites were chosen for seed analysis (Kalkee in Victoria and Bellata in New South Wales) with replicates pooled to provide a composite sample for analysis. Seed from the Bellata trial were also crushed to produce seed meal for feed testing.

Samples for vegetative tissue analysis were obtained from 38 day old Event 26 and 33 day old Event 40 vegetative tissue from block plantings undertaken in Kununurra, Western Australia.

Post-harvest analysis

At each field trial site, seed was harvested from individual plots and kept separate. For the purposes of compositional analysis, replicates of each variety and Event were pooled to form a composite sample.

Composite seed samples were processed by grinding prior to being analysed. This was undertaken following the protocols provided by each of the testing laboratories.

Vegetative tissues were collected from bulk plantings and subjected to destructive analysis.

Table 8. Field trial sites used for composition analysis

Trial Site Number	Location	Dates	Trial Design	Material Collected	Analysis Undertaken
DIR121	Kununurra, Western Australia	Seed planted in May 2014, seed harvested in September 2014	RCB*	Seed (T4 generation)	Fatty acid profiling of SHOSO
DIR131 Site 6	Kununurra, Western Australia	Seed planted in April 2016, seed harvested in September 2016	Block Planting of Event 40	Seed (T7 generation)	Fatty acid profiling
DIR131 Site 7	Kununurra, Western Australia	Seed planted in April 2016, seed harvested in September 2016	Block Planting of Event 26	Seed (T7 generation)	Fatty acid profiling
DIR131 Site 8	Kalkee, Victoria	Seed planted in June 2016 and seed harvested in February 2017	RCB, 4 replicates	Seed; composite of 4 replicates (T7 generation)	Proximates, minerals, vitamins, amino acids, free sugars, anti nutritional components, fatty acid profile
DIR131 Site 10	Bellata, New South Wales	Seed planted in July 2016 and seed harvested in December 2016	RCB, 4 replicates	Seed; composite of 4 replicates (T7 generation)	Proximates, minerals, amino acids, free sugars, anti nutritional components, fatty acid profile
DIR131 Site 10	Bellata, New South Wales	Seed planted in July 2016 and seed harvested in December 2016	RCB, 4 replicates	Seed Meal; composite of 4 replicates (T7 generation)	Feed analysis
DIR131 Site 14	Kununurra, Western Australia	Seed planted in May 2017, tissue harvested in June 2017	Block planting of Event 26	Seed, 38 day old vegetative tissue (T8 generation)	Fatty acid profiling of seed; Proximates, anti-nutritional components and feed analysis
DIR131 Site 15	Kununurra, Western Australia	Seed planted in May 2017, tissue harvested in June 2017	Block Planting of Event 40	Seed, 33 day old vegetative tissue (T8 generation)	Fatty acid profiling of seed; Proximates, anti-nutritional components and feed analysis

* RCB: Randomised Complete Block

Statistical analysis

Event 26 and Event 40 characteristics were first compared to the advanced breeding line M1582 and subsequently to commercial safflower varieties. Data analysis was performed using SPSS Statistics (version 23) with the significance of differences between means tested using the Least Significant Difference (LSD) test ($p<0.01$). 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.

Combined literature ranges and tolerance intervals (Vardeman 1992) were also determined to ensure that components of Event 26 and Event 40 were within the normal range of conventional safflower varieties. Literature data were obtained from published sources (e.g. Rahamatalla et al., 1998, Ingale and Shrivastava 2011, Yu et al., 2013 and Al Surmi et al., 2016; Heuzé et al., 2016, Heuzé et al., 2017a; Heuzé et al., 2017b).

Compositional analysis of safflower seed

Nutritional analysis was conducted on safflower seed to confirm that the composition of Event 26 and Event 40 remained within the normal levels for safflower when compared to its parental control, M1582, and conventional non-transformed safflower. The compositional assessments determined the following concentrations:

1. Proximates
2. Minerals
3. Vitamins
4. Total amino acids
5. Fatty Acids
6. Anti-nutrients

Proximates

A summary of the proximate chemical composition of Event 26 and Event 40 seed compared with M1582 and commercial safflower seed are presented in Table 9.

Moisture Content

Moisture content is an important factor associated with seed storage quality and was significantly different ($p<0.05$) across the varieties tested. The moisture content ranged from 4.6 to 6.8%, consistent with the results of Ingale and Shrivastava (2011), Yu et al (2013) and Al Surmi et al., (2016) where the moisture content ranged from 5.5 to 7.4%.

M1582 that had the highest average moisture content (6.2±0.28%) compared to all other varieties, but was not significantly different ($p>0.01$) to Event 26 and Event 40, or other high oleic acid varieties. M1582 was significantly different ($p<0.01$) to the linoleic safflower variety Sironaria.

Event 26 (5.8±0.17%) and Event 40 (5.6±0.10%) were not significantly different ($p>0.01$) to any of the safflower varieties tested and mean values were within the literature range.

The mean differences observed may be related to differences in maturity at harvest, but all varieties were within the industry standards for moisture content (AOF 2017). It is recommended that for safe, long-term storage, threshed safflower seed should not exceed 8% moisture.

The analysis demonstrates that in relation to moisture content of harvested seed Event 26 and Event 40 are comparable to the conventional safflower lines tested.

Crude Protein

Protein content varied from 16-21% across all of the varieties investigated. There were no significant differences (p>0.01) in crude protein content and all were within the literature range. The analysis demonstrates that crude protein levels in Event 26 and Event 40 are comparable to the conventional safflower lines tested.

Crude Fat

Crude fat contents of Event 26 and Event 40 were not significantly different (p>0.01) to the control, M1582 or several of the commercial safflower varieties. The high oleic variety S-317 was significantly different (p>0.01) to Montola 2003 and Sironaria. This demonstrates that crude fat levels in Event 26 and Event 40 are comparable to the conventional safflower lines tested.

Ash

The analysis demonstrated that in relation to ash content, there were no significant differences (p>0.01) between Event 26 and Event 40 and the conventional safflower lines tested.

Carbohydrates

The total carbohydrate level was calculated by difference using the fresh weight derived data and the following equation: % carbohydrates = 100% - (% protein + % fat + % moisture + % ash). No significant differences in carbohydrate content (p>0.01) were observed between Event 26 and Event 40 and the conventional safflower lines tested.

Table 9. Proximate analysis of safflower seed

Characteristic*	Event 26	Event 40	M1582	S-317	Montola 2003	Sironaria	Tolerance Interval**	Literature Range***
Moisture	5.8±0.17^{ab}	5.6±0.10^{ab}	6.2±0.27^{a}	5.5±0^{ab}	5.85±0.18^{ab}	5.1±0.18^{b}	2.42–8.50	5.5–7.4
Crude Protein	20.1±2.55^{a}	21.8±1.13^{a}	21.5±4.24^{a}	16.2±0.0^{a}	19.5±9.69^{a}	18.5±4.45^{a}	0–39.10	14.7–37.2
Total Lipid/Fat	34.6±1.35^{ab}	34.7±1.39^{ab}	31.2±1.25^{ab}	30.5±1.22^{a}	37.7±1.51^{b}	37.7±1.52^{b}	1.0–60.0	13.7–29.0
Carbohydrates	35.85±3.64^{a}	34.2±2.76^{a}	37.7±4.75^{a}	45.1±1.55^{a}	34.29±8.77^{a}	36.72±4.99^{a}	3.60–66.70	22.5–48.9
Ash	3.1±0.28^{a}	3.19±0.30^{a}	2.9±0.20^{a}	2.7±0.33^{a}	3.3±0.28^{a}	2.2±0.24^{a}	0.50–6.60	3.4–4.2
Energy (calories/100g)	535.2±5.08^{ab}	536.3±4.64^{ab}	517.52±4.21^{a}	519.8±4.95^{a}	554.3±5.90^{bc}	559.9±6.21^{bc}	283–696	490–507

* Calculated by differences on a dry weight basis (g/100g) unless otherwise stated; Values are means ± standard error. Means with the same letter are not significantly different ($p>0.01$).
** Tolerance intervals were calculated to contain, with 95% confidence, 99% of the values in the population (Vardeman, 1992). Negative values were corrected to zero.
*** Combined literature ranges from Rahamatalla et al., (1998), Ingale and Shrivastava (2011), Yu et al., (2013) and Al Surmi et al., (2016)

Energy

Calories were calculated using the following equation: Calories (Kcal/100 g) = (4 × % protein) + (9 × % fat) + (4 × % carbohydrates). The energy potential of Event 26 and Event 40 were not significantly different ($p>0.01$) to the control, M1582 or several of the commercial safflower varieties. Some differences were observed between conventional safflower lines tested. Analysis demonstrates that the caloric potential for Event 26 and Event 40 are comparable to the conventional safflower lines tested.

Summary of Proximate analysis

Proximate analysis was undertaken on seed from Event 26 and Event 40 and compared to the parental control and conventional safflower. The analysis demonstrates that both Event 26 and Event 40 are comparable to their parental line M1582 and the conventional safflower lines tested.

Mineral composition of safflower seed

A number of minerals are essential plant nutrients. Some are required in larger amounts (macro nutrients) and some only in trace amounts (micro nutrients). Both macro- and micro- nutrients were analysed in seed samples from Event 26 and Event 40 and compared with M1582 and commercial safflower varieties (Table 10). Across the 9 minerals assayed, Event 26 and Event 40 were not significantly different ($p>0.01$) to the parental control M1582 and were comparable to the conventional safflower lines tested. Mineral contents for Event 26 and Event 40 were within the literature range for safflower.

Table 10. Mineral composition of safflower seed

Mineral*	Safflower Varieties					
	Event 26	Event 40	M1582	Montola 2003	Sironaria	Literature Range**
P	525±35^{ab}	530±60^{ab}	550±40^{ab}	735±5.0^{b}	390±20^{a}	330-770
Ca	215±15^{a}	220±20^{a}	205±5^{a}	215±15^{a}	200±40^{a}	59.0-270
Fe	6.25±0.35^{a}	5.95±0.65^{a}	5.95±0.45^{a}	6.1±0.8^{a}	5.65±1.1^{a}	3.53-42.2
Mg	245±5.0^{a}	245±15^{a}	245±5.0^{a}	315±5.0^{b}	200±10^{a}	30.55-320
K	710±50^{a}	755±45^{a}	650±70^{a}	695±85^{a}	525±85^{a}	156-780
Na	4.3±0.8^{a}	4.8±0.20^{a}	4.2±2.2^{a}	4.8±1.3^{a}	3.6±1.2^{a}	2.0-39.3
Cu	1.3±0.1^{a}	1.09±0.31^{a}	1.4±0^{a}	1.55±0.05^{a}	1.2±0.10^{a}	0.31-2.4
Mn	2.0±0.2^{a}	2.2±0.30^{a}	1.6±0.2^{a}	1.6±0.10^{a}	1.75±0.35^{a}	0.26-2.1

* Calculated by differences on a dry weight basis (mg/100g); Values are means ± standard error. Means with the same letter are not significantly different ($p>0.01$).
** Combined literature ranges from Heuzé et al., (2015) and Al Surmi et al., (2016) and non-GM controls from this study.

Vitamin analysis of safflower seed

Vitamin analysis was undertaken from composite samples from two trial sites. The levels of many of the Vitamins examined in Event 26 and Event 40 were comparable to the parental control (M1582) and conventional safflower lines tested. Levels of Vitamin B1 and B6 were variable within Event 26 and Event 40. Vitamin B6 was considerably higher across safflower samples from this study compared to those reported in the literature for safflower and for other oilseeds (Table 11).

Vitamin B6 functions as a cofactor of many enzymes. In particular, pyridoxal 5′-phosphate, which is the active form of Vitamin B6, has multiple roles as a versatile cofactor of enzymes that are mainly involved in the metabolism of amino acid compounds (Grogan 1988; Rottmann et al. 1991; Helmreich 1992; Mihara et al. 1997; Kack et al. 1999). The data suggests that safflower *per se* may be a good source of Vitamin B6.

Amino acid analysis of safflower seed

A total of 16 amino acids were examined (Table 12). No significant differences ($p>0.01$) were observed between Event 26 and Event 40 with any of the conventional safflower lines tested. The analysis demonstrates that the amino acid composition of Event 26 and Event 40 are comparable to the parental control M1582 and conventional safflower.

Comparing the amino acid data of the safflower varieties to other oilseeds (canola, cotton and soybean) indicates that safflower is, on average, lower in the levels of essential amino acids, particularly lysine, methionine and threonine. These are important components of animal feed supplements.

Table 11. Vitamin analysis of safflower seed

Vitamin	Safflower Varieties							Oilseeds****	
	Event 26	Event 40	M1582	S-317	Montola 2003	Sironaria	Literature Comparison***	Canola	Soybean
Ascorbic Acid (Vitamin C)*	<1.0[a]	<1.0[a]	<1.0[a]	<1.0	<1.0	<1.0	0.0	N/A	N/A
Thiamine (B1)*	9.33±8.67[a]	10.32±9.86[a]	1.04±0.36[a]	0.60	0.49	0.60	1.163	0.916	0.36
Riboflavin (B2)*	0.06±0[a]	0.08±0.04[a]	0.08±0.02[a]	0.09	0.06	0.04	0.415	0.446	0.42
Niacin (B3)*	<1.0[a]	<1.0[a]	<1.0[a]	<1.0	<1.0	<1.0	2.284	14.37	2.6
Panthothenate acid (B5)*	1.46±0.95[a]	2.6±0.3[a]	2.65±0.35[a]	3.1	1.5	1.6	4.0	0.757	1.3
Vitamin B6*	7.1±0.3[a]	13±1.0[a]	4.68±4.42[a]	6.7	7.9	5.9	1.17	0.639	0.544
Folic Acid**	<0.1[a]	<0.1[a]	<0.1[a]	<0.1	<0.1	<0.1	0.0	7.57	5.16
Vitamin B12**	<0.04[a]	<0.04[a]	<0.04[a]	<0.04	<0.04	<0.04	0.0	N/A	N/A
Vitamin A**	<5.0[a]	<5.0[a]	<5.0[a]	<5.0	<5.0	<5.0	3.0 RAE	N/A	N/A
Vitamin D2**	<0.5[a]	<0.5[a]	<0.5[a]	<0.5	<0.5	<0.5	0.0	N/A	N/A

* Calculated by differences on a dry weight basis (mg/100g)
** Calculated by differences on a dry weight basis (µg/g)
*** USDA National Nutrient Database for Standard Reference (Release 28, released September 2015, slightly revised May 2016)
**** ILSI, 2016. International Life Sciences Institute Crop Composition Database, Version 6, www.cropcomposition.org [retrieved 21 June 2017]. Means for seed from all countries and all years.

Table 12. Amino acid analysis of safflower

Amino Acid*	Safflower Variety						Oilseeds****		
	Event 26	Event 40	M1582	Montola 2003	Sironaria	Literature Range***	Canola	Cotton	Soybean
Histidine	5.35±0.03[a]	5.08±0.23[a]	5.0±0.64[a]	4.75±1.1[a]	4.50±0.25[a]	2.8–12.3	7.48	6.96	10.47
Serine	9.13±0.16[a]	8.65±0.29[a]	8.33±0.94[a]	7.98±1.6[a]	7.58±0.80[a]	0.09-16.10	11.4	10.96	20.04
Arginine	18.93±0.23[a]	17.78±0.68[a]	16.85±2.25[a]	15.75±3.70[a]	15.18±1.78[a]	9.0–52.80	15.73	29.45	28.45
Glycine	11.48±0.17[a]	10.9±0.40[a]	10.45±1.24[a]	10.25±2.0[a]	9.78±1.06[a]	6.6–13.80	13.23	10.47	17.08
Aspartic Acid**	21.53±0.14[a]	20.05±0.73[a]	19.65±2.3[a]	18.25±4.16[a]	16.75±2.09[a]	2.01–32.30	18.98	23.31	45.6
Glutamic Acid**	43.15±0.50[a]	40.03±1.67[a]	37.68±4.6[a]	34.85±7.83[a]	35.33±4.23[a]	0.21–88.20	49.4	48.42	71.1
Threonine	6.5±0.09[a]	6.15±0.22[a]	5.85±0.69[a]	5.78±1.06[a]	5.45±0.58[a]	0.61–10.3	11.16	8.14	15.68
Alanine	8.68±0.08[a]	8.25±0.40[a]	7.95±-.98[a]	7.68±1.63[a]	7.05±0.81[a]	1.22–26.00	11.01	10.14	17.0
Proline	9.33±0.13[a]	8.73±0.58[a]	8.53±1.0[a]	7.65±1.62[a]	7.30±0.90[a]	0.10–29.50	15.8	9.53	19.83
Lysine	6.6±0.04[a]	6.23±0.29[a]	6.05±0.72[a]	6.0±1.13[a]	5.25±0.61[a]	3.90–46.90	15.7	11.23	25.6
Tyrosine	4.23±0.16[a]	4.10±0.10[a]	3.73±0.51[a]	3.78±0.85[a]	3.33±0.34[a]	2.20–23.20	6.84	7.5	13.65
Methionine	1.05±0.26[a]	1.15±0.26[a]	0.55±0.05[a]	0.65±0.09[a]	0.58±0.03[a]	0.40–30.01	4.79	3.76	5.41
Valine	11.03±0.09[a]	10.38±0.53[a]	10.03±1.31[a]	9.73±2.15[a]	9.1±1.19[a]	5.80–20.50	13.02	11.3	18.7
Isoleucine	7.93±0.10[a]	7.43±0.34[a]	7.23±0.91[a]	7.03±1.52[a]	6.48±0.80[a]	4.30–28.10	10.3	8.37	18.0
Leucine	13.65±0.15[a]	12.8±0.55[a]	12.3±1.53[a]	11.85±2.51[a]	11.3±1.33[a]	7.30–39.20	18.2	14.72	29.7
Phenylalanine	9.23±0.10[a]	8.75±0.38[a]	8.4±1.04[a]	8.1±1.76[a]	7.63±0.88[a]	4.90–31.50	11.11	13.57	20.1

* Calculated based on free amino acid molecular weight (mg/g DW). Values are means ± standard error (n=4), composite samples from two independent field trials. Means with the same letter are not significantly different ($p<0.01$)
** As Asparagine is hydrolysed to Aspartic Acid and Glutamine to Glutamic Acid, the reported amounts of these acids is the sum of those respective components
*** Combined literature ranges included non-GM samples from this study, data from Ingale and Shrivastava (2011) and Al Surmi et al., (2016)
**** ILSI, 2016. International Life Sciences Institute Crop Composition Database, Version 6, www.cropcomposition.org [retrieved 21 June 2017]. Means for seed from all countries and all years (mg/g DW) .

Fatty acid profile from safflower seed

Safflower is an oilseed crop. Event 26 and Event 40 safflower have been genetically modified to accumulate super high levels of oleic acid in the seed. The fatty acid composition of field grown Event 26 and Event 40 were analysed for their fatty acid profiles and compared to conventional safflower (Table 13). As expected, in Event 26 and Event 40, the polyunsaturated fatty acid linoleic acid and monounsaturated oleic acid and the saturated fatty acid palmitic acid were significantly different ($p<0.01$) to the parental control M1582 and other safflower varieties (Table 13). Other fatty acid components remained comparable to the parental M1582 control and other safflower varieties.

Collectively, the fatty acid analysis of seed from Event 26 and Event 40 demonstrated the efficacy and specificity of the down regulation of the *CtFAD2.2* and *CtFATB* genes.

Table 13. Fatty acid composition of safflower seed

Fatty Acid Component*	Event 26**	Event 40**	M1582 High Oleic Type	S-317*** High Oleic Type	Montola 2003*** High Oleic Type	Sironaria*** Low Oleic Type
Palmitic acid C16:0	3.40±0.12a	3.34±0.25a	6.27±0.12b	4.76±0.13c	6.03±0.12bcd	7.33±0.06bd
Palmitoleic acid C16:1	0.08±0.03a	0.13±0.06a	0.03±0.01a	0.20±0.00a	0.20±0.00a	0.13±0.03a
Stearic acid C18:0	1.40±0.11a	1.71±0.11ab	1.76±0.08b	1.56±0.27ab	1.73±0.03ab	2.28±0.05ab
Oleic acid C18:1	92.47±0.26a	91.82±0.43a	75.90±0.58b	75.33±1.28b	76.00±0.68b	15.95±1.59c
Linoleic acid C18:2	2.43±0.20a	2.53±0.26a	15.71±0.58b	16.22±1.00b	14.33±0.69b	73.05±1.68c
Linolenic acid C18:3	0.01±0.00ab	0.00±0.00a	0.00±0.00a	0.03±0.02b	0.10±0.00c	0.10±0.00c
Arachidic acid C20:0	0.16±0.03a	0.21±0.03a	0.23±0.03a	0.36±0.04a	0.40±0.00a	0.35±0.03a

* Seed samples were analysed by NSW Department of Primary Industries Oil Testing Service and CSIRO. Fatty acid levels are presented as a percentage of total fatty acids (%). Values are means ± standard error. Means with the same letter are not significantly different (>0.01).

** Event 26 and Event 40 seed samples were obtained from field trials conducted under DIR131 in 2016 and 2017 from geographically diverse locations.

*** S-317, Montola 2003, M1582 and Sironaria are conventional safflower. Seed samples for analysis were obtained from NSWDPI and/or CSIRO. Seed samples were from agronomic field trials undertaken independently of DIR131 as well as from field trials conducted under DIR131 in 2016 from geographically diverse locations.

Analysis of free sugars from safflower seed

There were no significant differences (p>0.01) in free sugar levels between Event 26 and Event 40 compared to the conventional safflower lines tested (Table 14).

Table 14. Free sugars* in safflower

Event 26	Event 40	M1582	Montola 2003	Sironaria
0.94±0.37[a]	1.18±0.52[a]	1.31±0.49[a]	0.99±0.42[a]	0.82±0.11[a]

* Calculated by differences on a dry weight basis (g/100g)
** Means with the same letter are not significantly different (P>0.01)

Analysis of anti-nutrients in safflower seed

Anti-nutrients and phytochemicals found in foods have been categorised as having both adverse and beneficial health effects in humans and animals. Two anti-nutrients were examined in safflower Event 26 and Event 40, tannins and cyanide content.

Tannins

Tannins are polyphenols that can bind to and precipitate proteins (Chung et al 1998; Tadele 2015). In livestock diets, tannins may diminish weight gains, apparent digestibility and feed utilisation efficiency. These anti-nutritional effects have generally been attributed to inhibition by tannins of digestion of dietary proteins. Other effects associated with dietary tannin are systemic, requiring absorption of inhibitory material from the digestive tract into the body (Chung et al., 1998).

The levels of tannins in the safflower tested ranged from 0.08–0.41% (Table 15). Event 26 and Event 40 were not significantly different (p>0.01) to M1582 and the conventional lines tested. The levels in Event 26 and Event 40 were much lower than the levels reported by Ingale and Shriwastarva (2011), safflower varieties PBNS-12 and PBNS-40, having tannin concentrations of greater than 0.5%. Further, the levels in Event 26 and Event 40 are lower to that of other oilseeds (e.g. canola–1.5%, Canadian Canola Council (2015); rapeseed–0.5%, Heuzé et al. (2017a); soybean–0.85%, Heuzé et al (2017b); sunflower meal–1.4%, Heuzé et al., (2016)).

Table 15. Tannin content* of safflower seed

Safflower Variety				
Event 26	**Event 40**	**M1582**	**Montola 2003**	**Sironaria**
0.13±0.04[a]	0.18±0[a]	0.13±0.02[a]	0.41±0.21[a]	0.1±0.05[a]

* Calculated by differences on a dry weight basis (%)
** Means with the same letter are not significantly different (p>0.01)

Hydrogen Cyanide

Prussic acid, also known as hydrogen cyanide or HCN, is a chemical compound both useful and dangerous. Although it is naturally present in some plants, this substance can also be synthesised through a variety of chemical processes. It has been reported that 110 to 135 ppm may be fatal after 0.5 to 1 hour or later, or dangerous to life (Flury and Zernik 1931 in CDC 2017). The acute

lethal dosage of hydrogen cyanide (HCN) in most animal species is ~2 mg/kg with plant materials containing ≥200 ppm of cyanogenic glycosides considered dangerous (Cope 2017). On a dry weight basis, plant materials with 200-500 ppm HCN should be considered potentially toxic to livestock (Williams 2012).

All safflower varieties examined contained less than 2.5 ppm (mg/Kg; Table 16). The analysis demonstrates that Event 26 and Event 40 are comparable to the conventional safflower lines tested and that potential cyanide production among varieties is very low and not considered toxic to either humans or animals.

Table 16. Cyanide content* of safflower seed

Safflower Varieties					
Event 26	**Event 40**	**M1582**	**S-317**	**Montola 2003**	**Sironaria**
<2.5	<2.5	<2.5	<2.5	<2.5	<2.5

* Calculated by differences on a dry weight basis (mg/Kg)

Feed quality analysis of seed meal

Safflower seeds used for oil production may be either cold pressed, expeller pressed or solvent extracted (GRDC, 2010). The by-product, safflower meal, is mostly used as a protein ingredient for animal feeding. Safflower seed from a field trial undertaken at Bellata in 2016 were passed through an expeller and the resulting meal analysed for feed quality undertaken (Table 17). Feed analysis included measurements of several important quality attributes or parameters (e.g., crude protein, fibre, digestibility, etc.) that define the overall nutritive value.

The data represent an average composition for each of the factors. As the data did not indicate substantial differences between Event 26, Event 40 and the control MM1582, only one feed analysis was undertaken on the seed meal. Event 26 and Event 40 are comparable to the parental control M1582 and the conventional safflower lines tested.

Analysis of vegetative tissue

Safflower is a valuable forage for Mediterranean areas since it remains green and has a higher feed value under dry conditions (Stanford et al., 2001; Landau et al., 2005; Peiretti 2009). The potential value to Australian farming systems is poorly understood, however several reports indicate strategic use can offer satisfactory growth rates and productivity to livestock (French et al. 1988).

Safflower maybe a valuable forage provided it is harvested from mid-budding to early blooming stage so that it is not too prickly and thus remain highly palatable to livestock (Landau et al., 2005), however, there are also thornless cultivars, which may be useful for feeding (Oyen et al., 2007).

Table 17. Feed analysis of safflower seed meal

Component*	LOR**	Event 26***	Event 40***	M1582***	Montola 2003***	Sironaria***
Neutral Detergent Fibre (%)	10	60	58	56	52	60
Acid Detergent Fibre (%)	2	43	41	39	36	41
Crude Protein (%)	0.6	22.9	23.3	25.1	27.2	23.1
Crude Fat (%)	0.5	7.0	7.8	8.1	10.0	7.8
Dry Matter Digestibility (%)	39	49	49	52	55	49
Digestible Organic Matter in the Dry Matter (%)	38	49	49	52	55	49
Inorganic Ash (%)	1	3	3	3	4	3
Organic Matter (%)	1	97	97	97	96	97
Metabolisable Energy (MJ/Kg DM)	4.3	9.6	9.8	10.2	11.2	9.7
Aparent Metabolisable Energy Poultry (MJ/Kg DM)	5.8	6.8	7.2	7.5	8.6	7.0

* Results are on a dry matter basis (%; g/100g equivalent) or as otherwise stated.
** LOR = Limit of reporting, the minimum quantity that can be reported with confidence.
*** Composite seed samples from a field trial undertaken at Bellata in 2016 were passed through an expeller and the resulting meal analysed for quality.

Safflower can be directly grazed by sheep and cattle or fed fresh in a cut-and-carry system. Safflower is also used as hay especially if it has suffered from frost. It has been recommended that silage should be prepared from safflower at the budding stage (Peiretti, 2009; Oyen et al., 2007).

The SHOSO lines may present a forage opportunity, particularly during drought where seed remaining in the safflower stubble following harvest germinates on an early rainfall event, generating early vegetative growth available for livestock grazing in autumn. Therefore, the forage quality of Event 26 and Event 40 was examined on late vegetative tissue from block plantings in 2017 (Table 18). The majority of the components examined were within the literature ranges reported for vegetative safflower tissue. Of note, several components differed to those reported elsewhere. For example, the protein levels of Event 26 and Event 40 were much higher than reported in the literature. However, it is noted that protein levels are dependent on growth stage and agronomic conditions, with higher crude protein levels reportedly associated with nitrogen application (Danieli et al., 2011) and decreases in crude protein recorded through later stages of development (Corleto et al., 2005; Peiretti 2009).

The analysis of vegetative tissue was an opportunistic analysis undertaken on material obtained from a single field trial. Unfortunately, the trial did not have M1582 material growing at the same time as the trial contained pure homozygous material and the presence of non-GM safflower would have increased the likelihood of generating a heterozygous seed population. GO Resources considered undertaking analysis of glasshouse grown material (including other safflower varieties), however considers analysis of the field grown material more closely represents 'typical' vegetative tissue that might be grazed. The feed quality was compared to other safflower results published in the literature as well as other feedstocks (see Table 18). The feed analysis of Event 26 and Event 40 indicates that they are comparable to other safflower varieties.

Table 18. Feed quality analysis of Event 26 and Event 40 vegetative tissue

Component*	LOR**	Event 26	Event 40	Literature Range***
Moisture	0.5	89.75±0.05a	90.45±0.35a	91.7–97
Dry Matter	0.5	10.25±0.05a	9.55±0.35a	8.3–13
Neutral Detergent Fibre	10	45.5±3.5a	44.5±2.5a	29.5–49
Acid Detergent Fibre	2	23±1.0a	21.5±1.5a	17.2–35
Acid Detergent Lignin	0.5	11.4±1.2a	12.15±6.0a	N/A
Crude Fibre	2	15.5±0.50a	14.5±0.50a	18–36
Crude Protein	0.6	39.25±0.95a	40.4±0.7a	7.0–27
Crude Fat	0.5	2.15±0.05a	2.0±0.0a	N/A
Organic Matter	1	86.5±0.5a	86.5±0.05a	82.9
Dry Matter Digestibility	39	67.5±3.5a	66.5±0.50a	N/A
Digestible Organic Matter in the Dry Matter	38	64.0±3.0a	63.0±1.0a	N/A
Inorganic Ash	1	13.5±0.5a	13.5±0.5a	12–17
Metabolisable Energy (ML/Kg DM)	4.3	9.95±0.55a	9.8±0.10a	16.2

* Unless otherwise stated, results are on a dry matter basis (%; g/100g equivalent); Means with the same letter are not significantly different (p>0.01)
** LOR = Limit of reporting, the minimum quantity that can be reported with confidence.
*** Literature range (Landau et al., 2004; Corleto et al., 2005; Arslan et al 2008; Peiretti 2009; Danieli et al., 2011); N/A: data not available, single values from Peiretti 2009.

Anti-nutrients in vegetative tissue

Safflower forage contains tannins, however their effect on feed value has yet to be investigated in detail (Landau et al., 2005). The vegetative tissue of Event 26 and Event 40 were very low in tannin content (0.045±0.01% and 0.065±0.02% respectively). These levels would not be considered toxic and are significantly less than reported by Landau et al., 2005 (1.8-2.4%).

Literature suggests that forage (i.e. hay, green chop, silage, or growing plants) containing >220 ppm cyanide as HCN on a wet-weight (as is) basis are toxic as an animal feed. However, forage containing <100 ppm HCN, wet weight, is usually safe for grazing animals.

In contrast analyses performed on a dry-weight basis demonstrated that >750 ppm HCN is hazardous, 500–750 ppm HCN is doubtful, and <500 ppm HCN is considered safe for grazing animals.

Analysis of the vegetative tissue of Event 26 and Event 40 demonstrated that the HCN levels were lower than 2.5 ppm and are therefore unlikely to be toxic as a green forage grazed by livestock.

Summary of analysis of vegetative tissue

The analysis of late stage vegetative tissue from Event 26 and Event 40 suggest that forage could be considered non-toxic to livestock if grazed.

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

Safflower has a long history of safe use. Global production in 2014[5] was less than 0.8 million tonnes.

Safflower Event 26 and Event 40 provided in this submission have been transformed with T-DNA designed to down-regulate endogenous safflower genes and to produce the HPH protein. The introduction of the RNAi sequences and the HPH protein have no nutritional impact on the safflower events. This is supported by the fact that:

- Molecular characterisation demonstrated stability of the inserts during numerous generations
- The HPH protein has a history of safe consumption and no significant homology to known allergens and toxins; and
- Compositional analysis did not indicate biologically significant changes to the levels of nutrients in events compared to their conventional counterparts. Event composition is within the normal variation of safflower cultivars and varieties and is substantially equivalent to conventional safflower varieties.

The most important nutritional changes between Event 26, Event 40 safflower and their untransformed controls, relate to seed specific reduction in *CtFAD2* and *CtFATB* leading to increased oleic acid and decreased linoleic acid and palmitic acid content in the seed. Food products derived from Event 26 and Event 40 safflower are anticipated to be nutritionally equivalent to food products derived from other commercially available safflower with increased oleic acid content, except that Event 26 and Event 40 safflower is expected to be even higher in oleic acid and lower in saturated fat.

D. Other Information

Where a biotech food has been shown to be compositionally equivalent to conventional varieties, the evidence to date indicates that feeding studies will add little to the safety assessment and generally are not warranted (see, for example, Bartholomaeus et al., 2013; OECD, 2003; Herman and Ekmay, 2014).

The only polypeptide produced by the inserts in safflower Event 26 and Event 40 is HPH, used as a selectable marker during the transformation process. The hygromycin resistance gene is commonly used in gene technology and there is no evidence that the HPH protein is either toxic or allergenic (OGTR 2012). Furthermore, considering the compositional equivalence between each safflower event and conventional varieties, and the lack of any observed phenotypic characteristics indicative of unintended effects arising from the genetic modification process, there was no plausible risk hypothesis that would indicate the need for animal feeding studies.

[5] Food and Agriculture Organization of the United Nations, http://faostat3.fao.org; data retrieved 7th December 2017.

Appendix 1.

INFORMATION IN THIS APPENDIX IS COMMERCIALLY SENSITIVE

Full DNA sequence of Event 26

Full DNA Sequence of Event 40

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