Author/s (year)	[redacted]
Title (version Nº) EXPURGATED	**Inheritance of the Omega 3 Trait – DHA Canola (OECD ID NS-BSØØ27-4)**
Owner	**Nuseed Pty Ltd**
Date	**December 31, 2016**
Project	**Omega-3 canola**
Report Nº	**2016-019**
Testing Facility	**Nuseed Pty Ltd** **5 Ballinger Street,** **Horsham, Vic, 3400** **Australia**
Dates of Work	**Jun 2013 - Oct 2016**
Test method	**Not Applicable**
GLP	**No GLP**
Confidentiality	**Yes**

TITLE:
INHERITANCE OF THE OMEGA 3 TRAIT – DHA CANOLA (OECD ID NS-B5ØØ27-4)

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ABBREVIATIONS

ALA	α-Linolenic acid, $18:3^{\Delta 9,12,15}$
ATR	Atrazine resistance
CSIRO	Commonwealth Scientific and Industrial Research Organization
DHA	Docosahexaenoic acid, $22:6^{\Delta 4,7,10,13,16,19}$
DHA Canola	Event NS-B50027-4 (OECD Identifier)
DPA	Docosapentaenoic acid, $22:5^{\Delta 7,10,13,16,19}$
EPA	Eicosapentaenoic acid, $20:5^{\Delta 5,8,11,14,17}$
ETA	Eicosatetraenoic acid, $20:4^{\Delta 8,11,14,17}$
GC-FID	Gas chromatography-flame ionization detector
HET	Heterozygous
HOM	Homozygous
KASP	Kompetitive Allele Specific PCR
LC-PUFA	Long-Chain ($\geq C_{20}$) Polyunsaturated fatty acid
MMT	Million metric tons
NS-B5ØØ27-4	DHA canola OECD identifier
OA	Oleic Acid, $18:1^{\Delta 9}$
PCR	Polymerase chain reactionb
PPT	Phosphinothricin
SDA	Stearidonic Acid, $18:4^{\Delta 6.9.12.15}$
STDEV	Standard deviation
TT	triazine-tolerant
wt	Wild type
ω3	Omega-3 fatty acid

EXECUTIVE SUMMARY

In collaboration with the Commonwealth Scientific and Industrial Research Organization (CSIRO), Nuseed Pty Ltd has developed genetically modified canola event NS-B50027-4 (DHA canola), which contains DHA in the seed oil.

The purpose of this study is to confirm the trait stability for DHA canola across five generations. The omega-3 long-chain (≥C20) polyunsaturated fatty acids (ω3 LC-PUFA) EPA, DPA and DHA (eicosapentaenoic acid, 20:5ω3; docosapentaenoic acid, 22:5ω3; docosahexaenoic acid, 22:6ω3) are produced at different levels in DHA canola.

BC_1F_2 progeny populations derived from crossing the elite event DHA canola to six non-GM Nuseed parents segregated according to Mendelian inheritance for two independent loci based on both phenotype (i.e. % LC-PUFA) and genotype testing. [redacted]

F_2 populations derived from crossing DHA canola to a non-GM Nuseed parent segregated according to Mendelian inheritance for each loci (i.e. 1:2:1). The F_2 homozygous progeny for both locus inserts were grown to seed as part of the introgression program and shown to produce the expected percentage of LC-PUFA.

Observations of DHA in the seed oil expressed over seven generations of selfing of the DHA canola also confirm stability of this trait across a range of growing environments.

TITLE:
INHERITANCE OF THE OMEGA 3 TRAIT – DHA CANOLA
(OECD ID NS-B5ØØ27-4)

I. INTRODUCTION

The omega-3 long-chain (≥C20) polyunsaturated fatty acids (ω3 LC-PUFA) EPA, DPA and DHA (eicosapentaenoic acid, 20:5ω3; docosapentaenoic acid, 22:5ω3; docosahexaenoic acid, 22:6ω3, respectively) are widely recognised for their beneficial roles in human health, particularly those related to cardiovascular and inflammatory health. EPA, DPA and DHA are primarily sourced from wild-caught fish and algal oils, with algae being the primary producer in the marine food web. These sources are under pressure from increasing demand for ω3 LC-PUFA by aquaculture, nutraceutical and pharmaceutical applications. Additional sources of these fatty acids can be produced by engineering land-based oilseed crops to convert native fatty acids to marine-type ω3 LC-PUFA, which then accumulate in seed oil. Canola is a commonly grown oilseed with 67 million metric tons (MMT) of rapeseed produced globally in 2015/16[1].

In collaboration with the Commonwealth Scientific and Industrial Research Organization (CSIRO), Nuseed Pty Ltd has developed genetically modified canola event NS-B50027-4 (DHA canola), which accumulates significant amounts of DHA in the seed oil.

In this DHA canola, seven fatty acid desaturases and elongases were introduced to convert oleic acid (OA) to DHA in a single pathway expression vector (See Report Nº 2016-002). The Omega 3 trait is controlled by two independent locus inserts [redacted]

Six F_2 and six BC_1F_2 populations were used to study trait inheritance of DHA canola event NS-B50027-4 in different genetic backgrounds or elite lines.

[1] http://www.ers.usda.gov/data-products/oil-crops-yearbook/oil-crops-yearbook/#World Supply and Use of Oilseeds and Oilseed Products, 30 March 2016.

II. PURPOSE

The purpose of this study is to confirm Mendelian segregation of trait (by phenotype and genotype) and loci and confirm the trait stability for DHA canola, OECD ID NS-B50027-4, across five generations.

III. MATERIALS & METHODS

A number of crosses were made between the elite event, DHA canola and non-GM varieties. The six BC_1F_2 populations were developed from crosses between six elite, non-GM lines (04CC-024*5026W-41-12, CC05004*507L-03-4-X-12, CC06026-13-11, NC0013-X-X-10, ND0004B6 and NX0052-10) and NS-B50027-4 T3 line (B0050-027-18-20). F_1s were then backcrossed with each elite non-GM line (Recurrent Parent) to produced BC_1F_1, and the BC_1F_1 with heterozygous Locus A and Locus B T-DNA inserts (AaBb) were used to generate BC_1F_2 populations through selfing (Figure 1; Table 1).

Six F_2 populations were developed from crosses between six elite, non-GMO lines (ATR Wahoo, ATR Stingray, ATR Bonito, Jackpot TT, NT0252 and NT0272) and DHA canola T5 line (B0050-027-18-20-12-19); F_1s were then used to generate F_2 populations through selfing. The 12 F_2 populations were produced (six recurrent parents by each of the two loci) and were expected to be segregating for both Locus A and Locus B T-DNA inserts (Figure 2, Table 5).

For descriptive purpose, the [redacted] T-DNA inserts were denoted as "A" and "B" and wildtype counterparts were donated as "a" and "b" in this study, respectively. Chi-squared (χ^2) test (Griffiths et al. 2000) was used to check whether the segregation of the Locus A and Locus B T-DNA inserts in the F_2 populations fitted Mendelian inheritance patterns:

- 1 (AABB) : 1 (AAbb) : 2 (AABb) : 2 (AaBB) : 4 (AaBb) : 2 (Aabb) : 2 (aaBb) : 1 (aaBB) : 1 (aabb) for both T-DNA inserts (Tables 1);
- 1 (AA) : 2 (Aa) : and 1 (aa) for the Locus A T-DNA insert (Table 5); and
- 1 (BB) : 2 (Bb) : 1 (bb) for the Locus B T-DNA insert (Table 5).

For identification purposed colors were used for zygosity of lines.

- Red = wild type
- Orange = heterozygous for Locus B; wild type for Locus A
- Yellow = homozygous for Locus B; wild type for Locus A
- Green = other remaining variants heterozygous/homozygous for Locus A
- Blue = homozygous for Locus A; homozygous for Locus B

A. EXPERIMENT Nº 1

The elite event, DHA canola, was crossed and backcrossed to 6 recurrent parents. The F_1 progeny was crossed to the recurrent parents and selfed to produce BC_1F_2 generation progeny. Mendelian inheritance was measured by both the % LC-PUFA phenotype (five phenotypes expected) and genotype (nine genotypes expected).

B. EXPERIMENT Nº 2

The elite event, DHA canola, was crossed with a number of non-Omega 3 elite parents. The F_1 progeny selfed to produce F_2 generation progeny. Mendelian inheritance was determined for each locus separately by genotype.

C. EXPERIMENT Nº 3

Various homozygous materials covering T_3 through T_7 generations from glasshouses and field trials were analysed for the percentage of DHA in canola seed oil to confirm stability of the trait.

D. LOCUS ZYGOSITY PREDICTION

Progeny plant tissue was screened using digital PCR to estimate the locus copy number value based on specific genes (i.e. PPT, delta 6 desaturase) and markers (KASP assay) specific to each locus insert.

E. FATTY ACID PHENOTYPE PREDICTION

Progeny seed fatty acid was determined using solvent extraction, followed by simultaneous saponification and methylation and analysis by GC-FID. This involved using an in-house method whereby seed samples were crushed and the oil was extracted from a crushed seed subsample into solvent. The solvent was evaporated off under nitrogen and an oil subsample was diluted in a new solvent. An aliquot was reacted with Meth Prep II (a saponification / methylation reagent). Samples were heated at 40°C to speed up the reaction and then injected on GC-FID using a BPX-70 column for fatty acid determination. Fatty acids were calculated as % composition of the oil where the area of each fatty acid peak was determined as a percentage of the sum of all the fatty acid peaks in the chromatogram. The % of specific fatty acids were estimated for: (Palmitic acid, Stearic acid, Oleic & Cis-vaccenic, linoleic, Alpha linolenic acid (ALA), Arachidic and Stearidonic (SDA), Paullinic, Gondoic and Gadoleic acid, Erucic acid and Eicosatetraenoic (ETA), Eicosapentaenoic acid (EPA), Docosapentaenoic acid (DPA),

Docosahexaenoic acid (DHA). The % LC PUFA was calculated (EPA% + DPA% + DHA%).

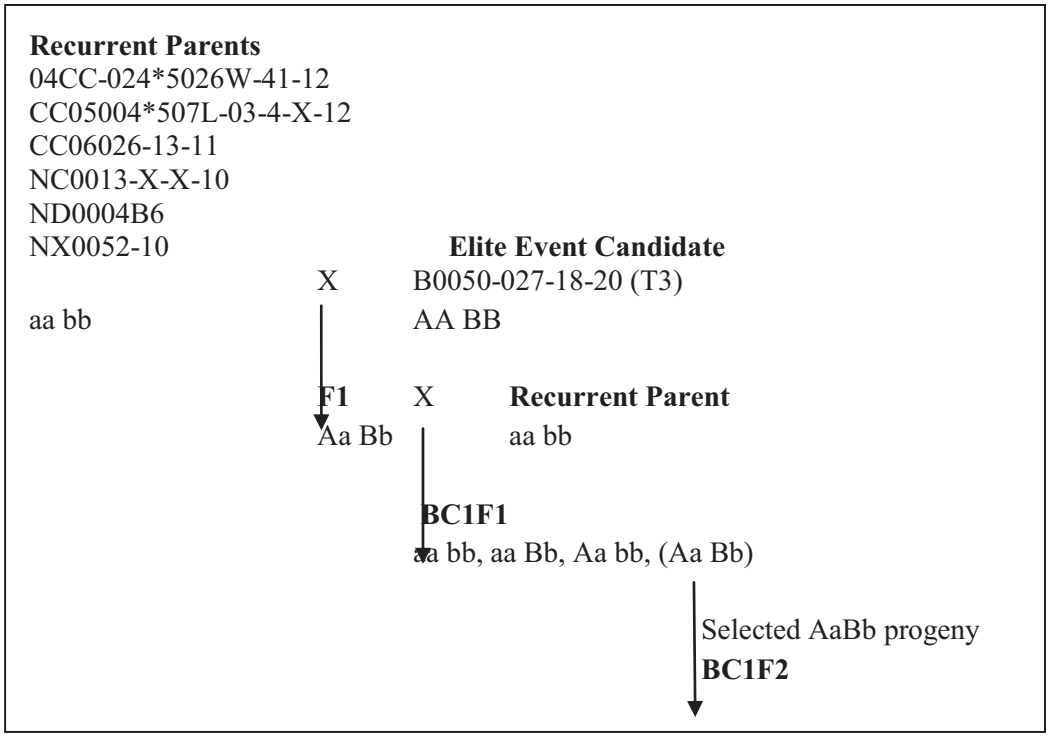

Figure 1. Schematic of crosses to produce BC_1F_2 progeny

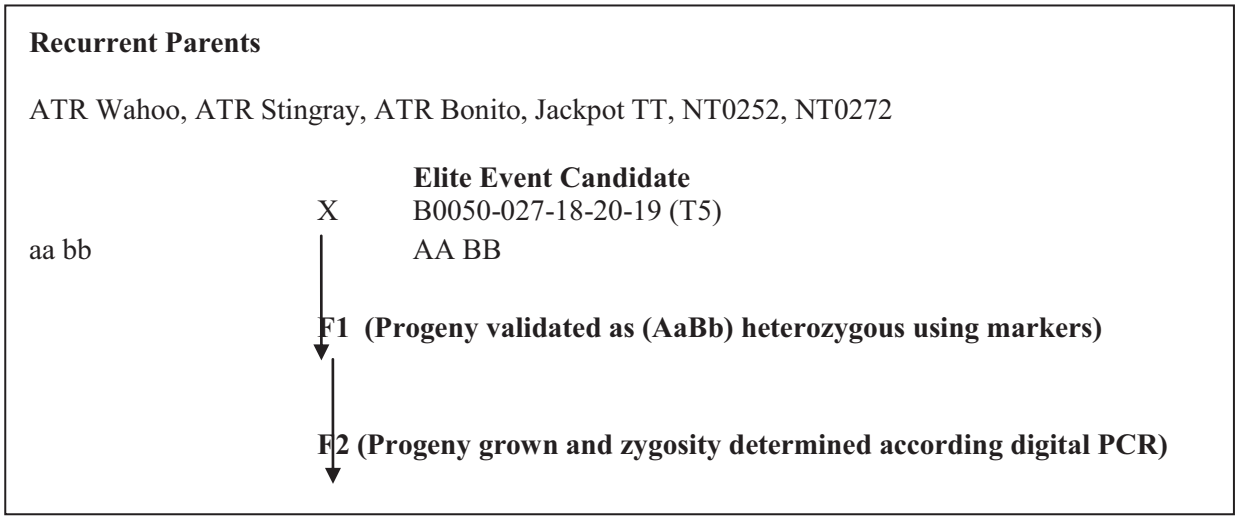

Figure 2. Schematic of crosses to produce F_2 progeny

IV. RESULTS & DISCUSSION

A. EXPERIMENT Nº 1

The phenotype and genotype results for Experiment 1 of the crosses described above in Section III, Materials & Methods, are presented in Tables 2 and 3, respectively. The segregation observed is the expected Mendelian inheritance pattern, with a Chi-squared value of 8.31 and 14.05 for % LC-PUFA and the expected/observed number of progeny (Tables 2 and 3). The mean and standard deviation of the nine phenotypes are presented in Table 4 and Figure 3. [redacted] The chi-squared test of 0.08 was observed in both analyses (Table 2 and 3).

Table 1. Expected two loci segregation of BC_1F_2 progeny

	A B	**Ab**	**aB**	**a b**
AB	AABB	AABb	AaBB	AaBb
Ab	AABb	AAbb	AaBb	Aabb
aB	AaBB	AaBb	aaBB	aaBb
Ab	AaBb	Aabb	aaBb	aabb

Table 2. Phenotype: Chi-squared test for hypothesis that % LC-PUFA phenotype of BC_1F_2 progeny fits the expected Mendelian inheritance segregation pattern.

Genotype	**Locus A**	**Locus B**	**Expected % of progeny**	**Expected Observable Phenotype % LC PUFA**	**Expected number of progeny**	**Observed number of progeny**
Aabb	wt	wt	0.0625	0	7.4	15
aaBB	wt	Hom	0.0625	<1	7.4	7
aaBb	wt	Het	0.125	<1	14.9	15
Aabb	Het	wt	0.125	>1 -7.5	81.8	75
AaBb	Het	Het	0.25	>1 -7.5		
AaBB	Het	Hom	0.125	>1 -7.5		
AAbb	Hom	wt	0.0625	>1 -7.5		
AABb	Hom	Het	0.125	>1 -7.5		
AABB	Hom	Hom	0.0625	> 7.5	7.4	7

Chi-squared Value $\chi^2 = 8.31$
P (0.05) df: 4 = 9.49
Chi test = 0.08
Accept the hypothesis: Phenotype variation fits expected Mendelian inheritance.

Table 3. Genotype: Chi-squared test for hypothesis that the two loci genotypes of BC_1F_2 progeny fits the expected Mendelian inheritance segregation pattern.

Genotype	Locus A	Locus B	Expected % of progeny	Expected Observable Phenotype % LC PUFA	Expected number of progeny	Observed number of progeny
aabb	wt	wt	0.0625	0	7.1	12
aaBB	wt	Hom	0.0625	<1	7.1	7
aaBb	wt	Het	0.125	<1	14.1	15
Aabb	Het	wt	0.125	>1 -7.5	14.1	17
AaBb	Het	Het	0.25	>1 -7.5	28.3	35
AaBB	Het	Hom	0.125	>1 -7.5	14.1	14
AAbb	Hom	wt	0.0625	>1 -7.5	7.1	3
AABb	Hom	Het	0.125	>1 -7.5	14.1	6
AABB	Hom	Hom	0.0625	> 7.5	7.1	4

Chi Squared Value $\chi^2 = 14.04$
P (0.05) df: 8 = 15.51
Chi test = 0.08
Accept the hypothesis: Genotype variation fits expected Mendelian inheritance.

Table 4. Observed mean and STDEV for % LC-PUFA phenotype for each BC_1F_2 segregant genotype as determined by locus-specific digital PCR analysis.

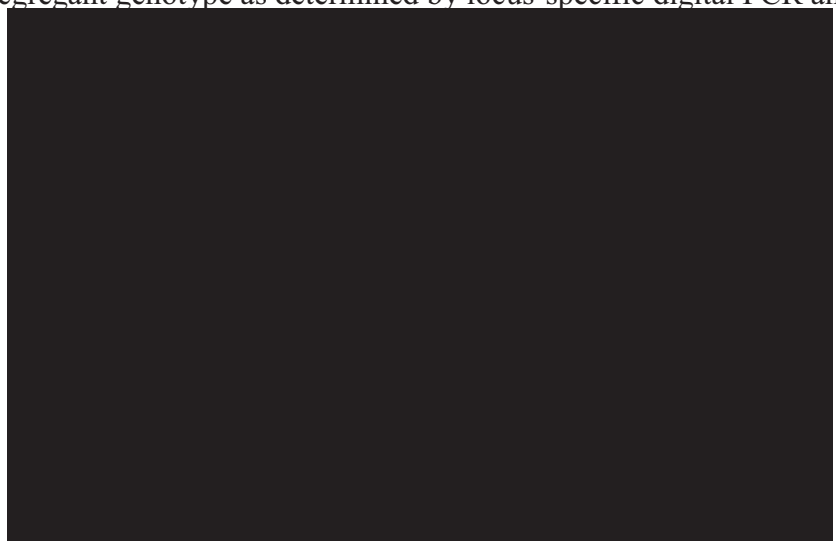

Figure 3. Observed mean and STDEV (error bars) for the % LC-PUFA phenotype for each BC_1F_2 genotype.

B. EXPERIMENT Nº 2

Twelve F_2 progeny lines were developed (six recurrent parents by each of the two loci). The genotype results for two recurrent parents (ATR Stingray and ATR Wahoo) are presented for Locus A in Table 6 (ATR Stingray) and Table 7 (ATR Wahoo), and for Locus B in Table 8 (ATR Stingray) and Table 9 (ATR Wahoo). These four results were representative of all the F_2 progeny lines. The results for the remaining eight F_2 progeny lines are not presented.

The segregation observed fits the expected Mendelian inheritance pattern, with a Chi-squared value of 2.25 and 4.32 for Locus A and 2.39 and 4.42 for Locus B.

The homozygous F_2 progeny for Locus A and Locus B were grown out and F_3 pooled seed data is presented in Table 10, showing a consistent high level of % LC-PUFA.

Table 5. Expected single locus segregation for F_2 progeny (1:2:1)

	A	a
A	AA	Aa
a	Aa	aa

	B	b
B	BB	Bb
b	Bb	bb

Table 6. Locus A (ATR Stingray): Chi-squared test for hypothesis that the Locus A genotype of F_2 progeny fits the expected Mendelian inheritance segregation pattern (ATR Stingray / B0050-027-18-20-12-19).

Pedigree	Zygosity	F_2 Locus A Observed	F_2 Locus A Expected 1:2:1
ATR Stingray / B0050-027-18-20-12-19	Hom	13	16
	Het	38	32
	wt	13	16
		64	64

Chi Squared Value $\chi 2 = 2.25$
P (0.05) df: 2 = 5.99
Chi test = 0.32
Accept the hypothesis: Genotype variation fits expected Mendelian inheritance.

Table 7. Locus A (ATR Wahoo): Chi-squared test for hypothesis that the Locus A genotype of F_2 progeny fits the expected Mendelian inheritance segregation pattern (ATR Wahoo / B0050-027-18-20-12-19).

Pedigree	Zygosity	F_2 Locus A Observed	F_2 Locus A Expected 1:2:1
ATR Wahoo / B0050-027-18-20-12-19	Hom	9	16
	Het	38	31
	wt	15	16
		62	62

Chi Squared Value $\chi 2 = 4.32$
P (0.05) df: 2 = 5.99
Chi test = 0.12
Accept the hypothesis: Genotype variation fits expected Mendelian inheritance.

Table 8. Locus B (ATR Stingray): Chi-squared test for hypothesis that the Locus B genotype of F_2 progeny fits the expected Mendelian inheritance segregation pattern (ATR Stingray / B0050-027-18-20-12-19).

Pedigree	Zygosity	F_2 Locus B Observed	F_2 Locus B Expected 1:2:1
ATR Stingray / B0050-027-18-20-12-19	Hom	13	16
	Het	38	31
	wt	13	16
		64	62

Chi Squared Value $\chi 2 = 2.39$
P (0.05) df: 2 = 5.99
Chi test = 0.30
Accept the hypothesis: Genotype variation fits expected Mendelian inheritance.

Table 9. Locus B (ATR Wahoo): Chi-squared test for hypothesis that the Locus B genotype of F_2 progeny fits the expected Mendelian inheritance segregation pattern (ATR Wahoo / B0050-027-18-20-12-19).

Pedigree	Zygosity	F_2 Locus B Observed	F_2 Locus B Expected 1:2:1
ATR Wahoo / B0050-027-18-20-12-19	Hom	10	16
	Het	39	31
	wt	13	16
		62	62

Chi Squared Value $\chi 2 = 4.42$
P (0.05) df: 2 = 5.99
Chi test = 0.11
Accept the hypothesis: Genotype variation fits expected Mendelian inheritance.

Table 10. % LC-PUFA in the F_2 derived F_3 pooled seed for plant genotypes homozygous for both inserts.

[redacted]

C. EXPERIMENT Nº 3

Observations of the percentage DHA in seed oil expressed over seven generations of selfing of DHA canola, NS-B50027-4, (Table 11) confirm stability of this trait for a range of growing environments. The candidate line is shown to be fixed and stable from T_3 – T_7 (five generations).

Table 11. Expression of the trait: seed oil % DHA over seven generations of selfing from the T_1 to the T_7 generation of NS-B50027-4.

	Line	Seed % DHA	Seed Sample	Experiment Location	Geographical Location
T1	**B0050-027**	[redacted]	Single Plant Pooled Seed	Glasshouse	Horsham
T2	**B0050-027-18**	[redacted]	Single Plant Pooled Seed	Glasshouse	Horsham
T3	**B0050-027-18-20**	[redacted]	Single Plant Pooled Seed	Glasshouse	Horsham
T3-X	**B0050-027-18-20-X**	[redacted]	Bulk	Field	Nurrabiel: Winter 2014
T3-X-X	**B0050-027-18-20-X-X**	[redacted]	Bulk	Field	Colac: Summer 2014-15
T3-X-X-X	**B0050-027-18-20-X-X-X**	[redacted]	Bulk	Field	St Helens Plains Winter
T4	**B0050-027-18-20-12**	[redacted]	Single Plant Pooled Seed	Glasshouse	Horsham
T5	**B0050-027-18-20-12-19**	[redacted]	Single Plant Pooled Seed	Glasshouse	Horsham
T5-X	**B0050-027-18-20-12-19-X**	[redacted]	Bulk	Field	Nurrabiel: Winter 2015 (Tent 1)
T5-X-X	**B0050-027-18-20-12-19-X-X**	[redacted]	Bulk	Field	Colac: Summer 2015-16
T6	**B0050-027-18-20-12-19-10**	[redacted]	Single Plant Pooled Seed	Glasshouse	Horsham
T6-X	**B0050-027-18-20-12-19-10-X**	[redacted]	Bulk	Field	Colac: Summer 2015-16 (Tent 1)
T7	**B0050-027-18-20-12-19-10-309**	[redacted]	Single Plant Pooled Seed	Glasshouse	Horsham

V. CONCLUSIONS

Two independent locus inserts are associated with the production of ω3 LC-PUFA in the seed. The results confirm that the LC-PUFA trait in seed oil follows a Mendelian inheritance pattern as expected for two independent loci contributing to expression. [redacted] Pooled seed samples from selfing of the DHA canola over seven generations of individual plants and bulk seed lots show that the seed oil percentage of DHA is highly stable for a wide range of growing environments.

VI. REFERENCES

Griffiths, AJF, Miller, JH, Suzuki, DT, Lewontin, RC, Gelbart, WM. 2000. *An Introduction to Genetic Analysis.* 7th edition. W. H. Freeman, New York.