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Genetic control of lutein esterification in wheat (*Triticum aestivum* L.) grain

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ABSTRACT

Lutein is a naturally occurring plant carotenoid compound that is also an important micro-nutrient for humans. While the biosynthesis of lutein in bread wheat (*Triticum aestivum* L.) is well understood, in contrast, there is little information on the mechanism or the genetic control involved in conversion of lutein to lutein esters during storage of wheat grain. The purpose of this research was to investigate the genetic control of lutein ester formation in wheat.

Lutein esterification varied between accessions of bread wheat, some related species and even grain tissues. A single locus controlling lutein esterification (designated *Lute*) was mapped on the short arm of chromosome 7D using a mapping population derived from zero-ester and high-ester parents. A GDSL-like lipase gene was identified in a syntenic region of the rice genome and wheat sequences with similarity to that gene were genetically mapped at the same position as *Lute*.

Since lutein esters seem to be more stable than free lutein, improved understanding of the genetics and biochemistry of lutein esterification could be useful for optimising the retention of lutein during the storage, handling and processing of wheat and other products that contain this important micronutrient.

1. Introduction

Lutein is an endogenous plant pigment that is located in the plastid membranes of plants and is also an important micro-nutrient for humans.

In wheat (*Triticum* spp.) grains, lutein is present in the seed coat, the starchy endosperm and the embryo. It represents 80–90% of total carotenoid content and contributes to the pale creamy to yellow colour of wheat-based end products. A white or pale creamy colour is preferred for bread and white noodles (eg. udon noodles), while a stronger yellow colour is preferred for most pasta products and for yellow alkaline noodles.

In harvest-ripe wheat grain, lutein is present primarily as free lutein. It is prone to oxidative degradation during storage and oxidation involving lipoxygenase during processing (Leenhardt et al., 2006).

During post-harvest storage, lutein may be converted to fatty acid esters, which appear to be more stable than free lutein (Subagio et al., 1999; Ahmad et al., 2013), by substitution of fatty acids at one or both hydroxyl groups at the ends of the lutein molecule. Lutein esters are de-esterified by intestinal enzymes prior to absorption of lutein into the blood stream (Alves-Rodriguez and Shao, 2004). Esterification appears to be favoured by storage under warm, low humidity conditions (Kaneko et al., 1995). In a survey of 138 hexaploid wheat cultivars (mostly from Japan), only nine, all of which are related by pedigree, did not form esters (Kaneko and Oyanagi, 1995).

The inhibition of lutein ester formation at higher temperatures (Subagio et al., 1999; Kaneko et al., 1995) and the absence of lutein esters in some wheat cultivars are consistent with an enzymatic

List of abbreviations: BAC, bacterial artificial chromosome; DArT, Diversity Arrays Technology; HRM, high resolution melting; PCR, polymerase chain reaction; SSR, simple sequence repeat.

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mechanism under genetic control. It has been suggested that lutein esterification could involve an acylhydrolase (Kaneko et al., 1995), but this has not been confirmed and apart from one report (Howitt et al., 2009) of a QTL on chromosome 2B associated with this trait no genetic analysis results have been reported. This is in marked contrast to the biosynthesis of lutein itself, for which both the biosynthetic pathway and genetic basis of lutein synthesis are well understood (Mares and Campbell, 2001; Howitt et al., 2009).

The present study was conducted to investigate the genetic control of lutein esterification in the grain of bread wheat (*Triticum aestivum* L.).

2. Materials and methods

2.1. Plant materials

The plant materials used here included:

Sunco, a high-quality Australian hard wheat cultivar;

Indis, a line developed in South Africa (Marais and Marais, 1990) carrying a chromosome segment from *Thinopyrum distichum* containing a gene for leaf rust resistance as well as a gene for increased grain lutein content. Seed was supplied by Professor RA McIntosh, University of Sydney;

DM5685*B12, a high lutein line developed at the University of Adelaide (Ahmad et al., 2013).

Haruhikari, a Japanese wheat cultivar that does not produce lutein esters (Kaneko et al., 1995);

Sunco/Indis.82, derived by self-pollination from a single Sunco/Indis F_2 plant with high grain lutein concentration.

DM06.6, a population of 117 doubled haploid lines derived from the F_1 of a cross Haruhikari x Sunco/Indis.82. The doubled haploid lines were developed using wheat × maize hybridisation (Laurie and Bennett, 1986);

Chinese Spring nullisomic-tetrasomic hexaploid wheat lines for which seed was provided by Margaret Pallotta, University of Adelaide;

Seven current Australian cultivars (Jandaroi, Tjilkuri, Saintly, Caparoi, Kalka, Tamaroi, Hyperno) and one breeding line (WID22221) of durum for which seed was supplied by Shafiya Hussein and Rob Wheeler, South Australian Research and Development Institute;

Seven durum cultivars (Altar, Croc, Gan, Kamilaroi, Yallaroi, Wollaroi, and EGA Bellaroi) and two germplasm lines (ZDB04.164 and ZDB04.288), four accessions of *Triticum monococcum*, seven of *Aegilops tauschii* and 45 synthetic hexaploid wheats (developed by the CIMMYT by crossing durum varieties Altar, Croc or Gan with a range of accessions of *Ae. tauschii*). Seed of these accessions was obtained from the Australian Winter Cereals Collection, Tamworth, NSW, Australia.

2.2. Storage conditions for lutein esterification

Grain was stored at 37 °C in an airtight container over silica gel for at least 60 d prior to analysis for lutein and lutein esters.

2.3. Tissue location of lutein esters

Plants of Sunco, Indis and DM5685*B12 were grown together in a glasshouse under natural daylight and with limited temperature control via evaporative air conditioning. Grains harvested from these plants were dissected into embryo (including the scutellum), seed coat (pericarp and testa) and endosperm tissues.

2.4. Reciprocal crosses

Reciprocal crosses were made between DM5685*B12 and Haruhikari (DM5685*B12/Haruhikari and Haruhikri/DM5685*B12, where the first named parent in each cross is the maternal parent). Similarly, reciprocal crosses were made between two contrasting Haruhikari//Sunco/Indis.82 doubled haploid lines: DM06.6.045, high lutein esters, and DM06.6.132, no lutein esters.

2.5. Germplasm panel and nullisomic-tetrasomics

The accessions of *T. monococcum*, *Triticum turgidum* ssp. *durum*, *Ae. tauschii* and synthetic hexaploid wheat and the Chinese Spring nullisomic-tetrasomic lines, 4 plants of each, were grown together in a glasshouse. Grains from the 4 plants of each accession were pooled. Of the 42 nullisomic-tetrasomic lines, 35 (including at least one for each of the 21 chromosomes of wheat) yielded sufficient grain for analysis.

2.6. Mapping population

Two seeds of each Haruhikari//Sunco/Indis.82 doubled haploid line were sown in 10-cm square pots in a glasshouse in 2007. Sowing was carried out on two days, approximately one month apart, with assignment of lines to each sowing time based on the order in which they had been transferred from culture and seed became available. Grains harvested from these plants were used for a preliminary assessment of ester phenotype and as seeds for a subsequent field experiment. In 2008, each doubled haploid line was sown in one 1-m rows in a field experiment at Urrbrae, South Australia, Australia.

2.7. Extraction and quantification of lutein and lutein esters

For each sample to be evaluated, 2 sub-samples each of 2 g of grain were ground using a ball mill (RotoMix™, 3M ESPE, St. Paul, MN, USA) for 7 s. Samples were extracted with 10 ml of methanol/tetrahydrofuran (1:1 v/v) containing the antioxidant butylated hydroxytoluene (0.1% w/v; Sigma Aldrich, St. Louis, Mo., USA) and the internal standard β-apo-8′-carotenal (250 μg; Fluka-Biochemika, Buchs, Switzerland; 10% purity) in a temperature regulated shaker for 2 h at 28 °C. Samples were then centrifuged (Clements Medical Orbital 420) for 10 min at 3500 g. Supernatants were saved and residues were re-extracted with 8 ml of n-hexane for 2 h then re-centrifuged. Extracts were combined and dried in a vacuum concentrator (UNIVAPO 150H, UniEquip, Munich, Germany). To remove any water present after drying, the combined extracts were partitioned into 2 ml n-hexane three times. These n-hexane fractions were combined, dried and reconstituted with 1 ml methanol:tert-butyl methyl ether (MTBE) (1:1) for quantification by reversed phase high pressure liquid chromatography (RP-HPLC) according to the method of Breithaupt et al. (2002). Lutein and its esters were separated using a C30 reversed-phase column (Ultracarb 5 μm C30, 250 × 4.6 mm; Phenomenex, Pennant Hills, NSW, Aust.) protected by a C18 cartridge (Security Guard, 4 × 3 mm; Phenomenex) at a column temperature of 35 °C using a RP-HPLC (Hewlett Packard 1100; Agilent Technologies, Santa Clara, Calif., USA) equipped with a photodiode array detector. Separation was achieved by eluting with a combination of two solvents: solvent A (81:15:4 methanol/MTBE/water) and solvent B (90:10 MTBE/methanol). The flow rate was 1 ml/min and the following schedule was used: 100% A for 10 min, gradient to 50% A by 40 min, then to 100% B by 50 min and finally isocratic elution with 100% B for 10 min. To prepare the column for the next run, a gradient from 100% B to 100% A for 5 min was used followed by 100% A isocratic

for 5 min. Separation was monitored at 445 and 450 nm. Lutein (Sigma–Aldrich) was used as a standard for identification and quantification of lutein and lutein esters. Lutein ester concentrations were calculated as free lutein equivalents and expressed as a percentage of total trans-lutein (trans free lutein plus lutein esters). Solvents were obtained from Scharlau Chemie S.A., Sentminant, Spain.

2.8. Genetic mapping

Leaf tissue was sampled from one individual plant of each of the parental lines, Haruhikari and Sunco/Indis.82, and each of 117 Haruhikari//Sunco/Indis.82 doubled haploid lines. DNA was isolated from each sample using a midi-prep method (Pallotta et al., 2000). Diversity Arrays Technology (DArT) markers (Jaccoud et al., 2001) were assayed on the DNA samples by Triticarte Pty Ltd. (Australia) using version 3 of its wheat microarray.

Each polymorphic DArT marker was tested for association with lutein ester content using a chi-squared (χ^2) 2×2 contingency test of independence with 1 degree of freedom and $\alpha = 0.05$. Based on the previously mapped positions of DArT markers that were found to be associated with lutein ester content, potentially linked SSR markers were selected and assayed on the parents and a subset of lines. SSR markers that were found to be polymorphic were assayed on the remaining lines using multiplex-ready PCR technology (Hayden et al., 2008). For each DArT or SSR marker, the observed genotypic ratios among Haruhikari//Sunco/Indis.82 doubled haploid lines were tested for deviation from the expected 1:1 ratio using a χ^2 analysis with 1 degree of freedom and $\alpha = 0.01$. A genetic map was constructed using MapDisto version 1.7.0 (Lorieux, 2012) and drawn using the software MapChart (Voorrips, 2002).

Results from published wheat-rice comparative genomic analysis were used to identify a region of interest in the rice genome (Kawahara et al., 2013). That region was examined for genes of potential interest. The predicted amino acid sequence (Genbank: AAP22477.1) of rice gene *LOC_Os06g05550* was used as a tBLASTx query against UK454 Chinese Spring genomic sequence reads (Cereals Data Base [http://www.cerealsdb.uk.net/cerealgenomics/CerealsDB]; Wilkinson et al., 2012) housed in an in-house database at the Australian Centre for Plant Functional Genomics.

2.9. Marker design and assay methods

Genious Pro® version 6.1 (Biomatters, New Zealand) and Primer 3 (Koressaar and Remm, 2007; Untergrasser et al., 2012) were used to design primers based on annotated sequence of UK454 Chinese Spring Contig03415. Primer combinations were tested on Haruhikari, Sunco/Indis.82, 10 zero/low-ester lines, 10 high-ester lines, Chinese Spring and six group-7 nullisomic-tetrasomic lines (CS N7A-T7B, CS N7A-T7D, CS N7B-T7A, CS N7B-T7D, CS N7D-T7A and CS N7D-T7B). Each PCR amplification was performed in a 10 μl reaction volume, containing 75 ng of template DNA, 0.1 units of Taq DNA polymerase (BIOLINE Immolase), 200 nM of each primer, $1 \times Mg^{2+}$-free PCR buffer (BIOLINE Aust Pty Ltd, Alexandria, NSW, Australia); 1.5 mM of $MgCl_2$, 875 nM of dNTP mixture (1.25 mM each). The thermal cycling protocol consisted of incubation at 95 °C for 10 min followed by 40 cycles of denaturing at 94 °C for 30 s, annealing at the appropriate temperature (Supplementary file 6) for 30 s and extension at 72 °C for 30 s, and a final extension at 72 °C for 10 min. Cresol Red (3 μl per sample) was added and products were separated on a 1% agarose gel. SYBR®Safe DNA gel stain (0.25 ×, Life Technologies Australia Pty Ltd, Mulgrave, VIC, Australia) was used to visualise bands with ultraviolet transillumination.

For two primer pairs that revealed dominant (presence/absence) polymorphisms between Sunco/Indis.82 and Haruhikari, 114 Haruhikari//Sunco/Indis.82 doubled haploid lines were genotyped using agarose gel electrophoresis. For two primer pairs that amplified products from both Haruhikari and Sunco/Indis.82, amplicons were purified and sequenced from Chinese Spring, one zero/low-ester line and one high-ester line. Sequences were aligned using the software MAFFT Align (Katoh and Standley, 2013) to identify sequence polymorphisms between the Haruhikari-type and Sunco/Indis.82-type lines. Primers were designed to flank these polymorphisms and were used to genotype the mapping lines with high resolution melting (HRM) technology on a LightCycler® 480 (Roche Diagnostics, Indianapolis, IN, USA). The PCR protocol used for the amplification of the products was the same as described above, but with addition of 0.3 μl of SYTO®9 green fluorescent nucleic acid stain (Life Technologies Australia Pty Ltd, Mulgrave, VIC, Australia). The melting protocol involved 30 s at 95 °C, cooling to 2 °C below the annealing temperature of the primers for 30 s, ramping to 95 °C with 25 fluorescent acquisitions per degree Celsius, and 1 min at 37 °C. Results were analysed with melting curve genotyping and gene scanning methods implemented in LightCycler® 480 Software Version 1.5.

3. Results and discussion

3.1. Tissue location of lutein esters in wheat grains

Both lutein and lutein esters were quantified in embryo, seed coat and endosperm tissues dissected from grain harvested from glasshouse-grown plants of Sunco, the high-lutein line Indis and the high-lutein Sunco//Sunco/Indis derivative DM5685*B12. All three lines had high lutein concentrations in the embryo and seed coat and, as expected, the endosperm lutein concentration in Sunco was lower (less than 0.8 μg g^{-1}) than in Indis or DM5685*B12 (Table 1). The proportion of lutein esters (mono-plus di-esters), calculated as a percentage of the total lutein, varied significantly among grain tissues (Table 1). In all three lines, the percentage of the lutein that was esterified was negligible in the embryo (not detectable in Sunco and below 1% in the other two lines), but substantial in both the seed coat (up to 45%) and endosperm (up to 75%). Since it was not possible to prepare seed coat from dry grain that was completely free of starchy endosperm it is likely that the ester percent for seed coat is an over-estimate. Typical HPLC profiles of low and high ester samples of DM5685*B12 have been previously reported (Ahmad et al., 2013) and the identity of the individual lutein ester species confirmed by LC-MS (Soriano et al., 2008) (Supplementary file 1).

3.2. Lutein esters in F_1 grains from reciprocal crosses between high-ester and zero-ester parents

In F_1 seeds of crosses between high-ester and zero-ester parents (Haruhikari crossed with DM5685*B12 and the Haruhikari//Sunco/Indis.82 doubled haploid lines DM06.6.045 and DM06.6.132 crossed with each other), the proportion of lutein esters was intermediate between the parents, and was lower when a zero-ester line (Haruhikari or DM06.6*132) was used as the female parent than in the reciprocal crosses (Table 2). Similarly, in F_1 seed from crosses in which one parent (Haruhikari) had much lower lutein content than the other (DM5685*B12), the lutein concentration was lower when Haruhikari was used as the female parent. These results are consistent with the synthesis of both lutein and lutein esters being under the genetic control of the triploid endosperm, in which two of the three sets of chromosomes derive from the female parent. The proportion of lutein present as esters in DM5685*B12 was higher in this experiment due to a longer storage time allowing

Table 1
Total lutein (free lutein plus lutein esters) concentration in whole grain and grain fractions of Sunco, Indis and DM5685*B12 and distribution of lutein esters among grain fractions. Grain fractions: Grain (WG), germ (G), endosperm (En) and grain coat (GC).

Wheat line	Concentration of total lutein (μg g^{-1})				Total lutein % of total grain lutein			Total free lutein % of total grain free lutein			Total lutein esters % of total grain lutein esters		
	G	En	GC	WG	G	En	GC	G	En	GC	G	En	GC
Sunco	5.2	0.8	0.6	0.8	22	48	30	32	32	36	nd[a]	55	45
Indis	4.8	6	1.6	5.8	4	79	17	12	67	21	<1	75	25
DM5685*B12	5.2	6.2	2.6	6	4	73	23	10	63	27	<1	69	31

Note: Germ consists of embryo, scutellum and some residual grain coat.
[a] No lutein esters detected.

Table 2
Concentrations of lutein and lutein esters in F_1 wheat grains derived from reciprocal crosses.

Parent or F_1	Lutein (μg g^{-1})	Lutein esters (% of lutein)
DM5685*B12 (high-ester parent)	6.3 ± 0.2	93 ± 0.7
Haruhikari (zero-ester parent)	1.6 ± 0.1	0
DM5685*B12/Haruhikari F_1	4.1 ± 0.3	53 ± 0.5
Haruhikari/DM5685*B12 F_1	2.7 ± 0.2	15 ± 0.4
DM06.6*045 (high-ester parent)	6.8 ± 0.2	79 ± 0.7
DM06.6*132 (zero-ester parent)	5.0 ± 0.1	0
DM06.6*045/DM06.6*132 F_1	5.3 ± 0.4	76 ± 0.5
DM06.6*132/DM06.6*045 F_1	4.8 ± 0.2	53 ± 0.5

Note: The first-named parent in each cross was used as the female.

more time for ester synthesis.

3.3. Quantification of lutein esters in the grain of durum wheat, T. monococcum, Ae. tauschii and synthetic hexaploid wheat

Among 17 accessions of durum wheat (*T. turgidum* L. ssp. *durum (Desf.) Husn*; genomic constitution AABB) evaluated here, no lutein esters were detected. In contrast, lutein esters were detected in six of seven accessions of *Ae. tauschii* Coss. (donor of the D genome of hexaploid bread wheat) and in four accessions of *T. monococcum* L. (genomic constitution A^mA^m) (Table 3). Among 45 synthetic hexaploids derived from crosses between durum and *Ae. tauschii*, most had lutein ester contents above 40%, but five (all with different *Ae. tauschii* parents) had lutein ester contents below 5% (Supplementary file 2). Considering all of these results, it seems the genetic determinant of lutein esterification in bread wheat was contributed by *Ae. tauschii* and is located on the D genome. The presence of lutein esters in accessions of *T. monococcum* (A^mA^m) but not in *T. turgidum* (AABB) may indicate that the capacity to esterify lutein was present in their diploid common ancestor, but was retained in only some lineages.

Table 3
Concentration of lutein esters in accessions of *Aegilops tauschii* and *Triticum monococcum*.

Species	Accession number in the Australian winter cereals collection	Lutein esters (% of lutein)
Aegilops tauschii	AUS 23990	0
	AUS 23980	66 ± 1
	AUS 23986	20 ± 3.5
	AUS 24048	73 ± 2.2
	AUS 24062	48 ± 0.2
	AUS 24091	73 ± 1.3
	AUS 24092	69 ± 2.3
Triticum monococcum	AUS 90357	42 ± 0.5
	AUS 90416	27 ± 0.5
	AUS 90417	37 ± 0.2
	AUS 19844	27 ± 0.8

3.4. Lutein esters in nullisomic-tetrasomic lines

Among 35 nullisomic-tetrasomic lines of Chinese Spring wheat, only the two lines that are nullisomic for chromosome 7D (CS N7D-T7A and CS N7D-T7B) were found to lack the capacity to form lutein esters (Supplementary file 3). In each of the other nullisomic-tetrasomic lines evaluated, more than 40% of the lutein was present as mono- and di-esters. These results indicate that the lutein ester phenotype of Chinese Spring is conferred by a gene (or genes) located on chromosome 7D. Howitt et al. (2009) reported a QTL for lutein esterification on chromosome 2B, however, in this experiment the group 2 chromosome nullisomic-tetrasomic lines were very similar in ester forming capacity.

3.5. Lutein esters in Haruhikari//Sunco/Indis.82 doubled haploid lines

In grain harvested from a seed increase conducted in a glasshouse in 2007, the percentage of lutein present as lutein esters was 0 for Haruhikari and 93.4 for Sunco/Indis.82 and ranged from 0 to 95.0 for 117.

Haruhikari//Sunco/Indis.82 doubled haploid lines (Supplementary file 4). Among the doubled haploid lines, the distribution of phenotypic values seemed bimodal (Fig. 1A) but the presence of intermediate values made it difficult to confidently classify the lines into two distinct phenotypic classes. When the same lines were grown in a field experiment in 2008, the percentage of grain lutein present as lutein esters was 0 for Haruhikari, 90.8 for Sunco/Indis.82 and ranged from 0 to 92.7 for the doubled haploid lines (Supplementary file 4). The phenotypic distribution was discontinuous (Fig. 1B) with no lines having phenotypic values between 19.6 and 62.9%. This made it possible to classify the lines unambiguously as having low/zero (up to 19.6%) or high (62.9% or more) lutein ester (Fig. 1C). This classification was consistent with the ranking of lines based on the data from the 2007 experiment, in that all of the lines that were assigned to the low/zero ester class had less than 10% lutein ester in the 2007 experiment and all of the lines that were assigned to the high ester class had more than 15% lutein ester in the 2007 experiment. In both experiments, the mean value for the lines classified as high lutein ester was significantly higher ($P < 0.01$) than that for the lines classified as low/zero ester. There was some quantitative phenotypic variation among the lines within classes (especially within the high-ester class) but this was not consistent between experiments (Fig. 1C). Some of the differences between the results obtained in the two years might be due to conditions in the 2007 seed increase, in which some lines had to be sown later than others and there was also a risk of competition between semi-dwarf and tall lines. Further, prior to the 2008 field experiment, the post-harvest storage conditions required to

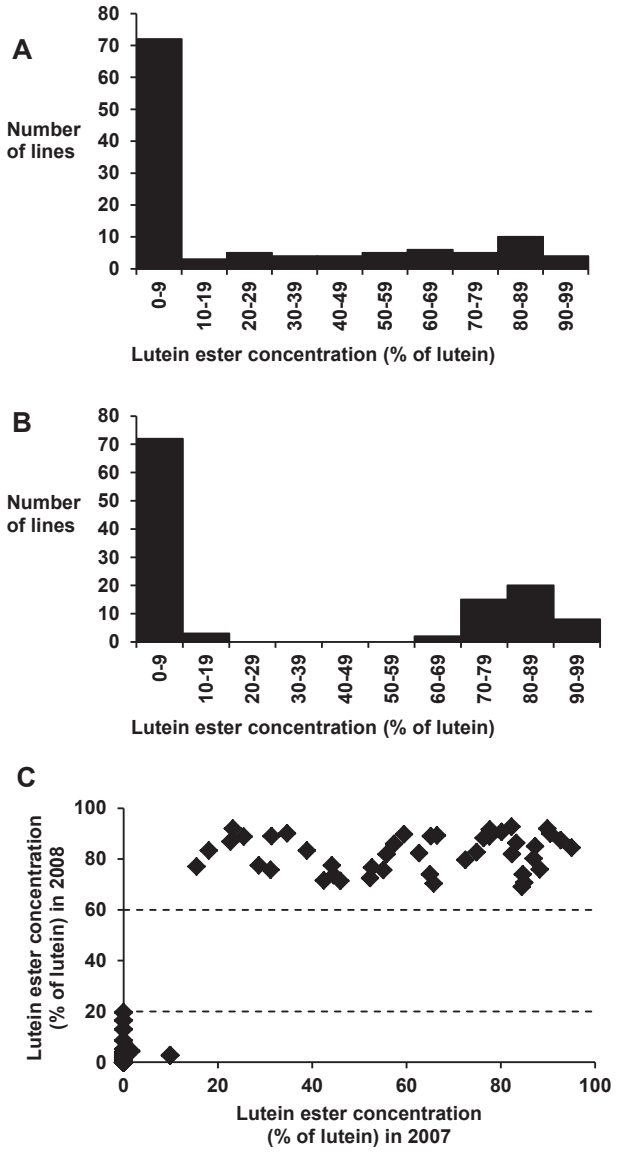

Fig. 1. Distributions of lutein ester concentrations among 117 Haruhikari//Sunco/Indis.82 doubled haploid lines. Lutein ester concentration was measured in grain harvested from a glasshouse experiment conducted in 2007 and in grain harvested from a field experiment conducted in 2008. (A) Frequency distribution of lutein ester concentration from the 2007 experiment. (B) Frequency distribution of lutein ester concentration from the 2008 experiment. (C) Lutein ester concentration from the 2008 experiment plotted against lutein ester concentration from the 2007 experiment.

optimise expression of the ester phenotype was optimised.

The observed phenotypic ratio between the low/zero- and high-ester phenotypic classes was 73:44. This deviates significantly ($P = 0.007$) from the 1:1 ratio expected for segregation at a single locus. One explanation for the excess of low/zero-ester lines could be preferential transmission of the Haruhikari chromosome through the doubled haploid process. The other parent, Sunco/Indis.82, contains an alien chromosome segment from *T. distichum* that replaces a large section of chromosome 7DL (Marais and Marais, 1990). Alien introgressions have previously been reported to cause frequency distortions in doubled haploid populations (Kammholtz et al., 2001). Despite the observed segregation distortion, the overall results from the Haruhikari//Sunco/Indis.82 population are consistent with the hypothesis that a locus, on chromosome 7D, which we designate *Lute* (*lut*ein *e*sterification), plays an essential role in formation of lutein esters in wheat.

Polymorphisms for two Diversity Arrays Technology (DArT) clones (*XwPt-1163* and *XwPt-3727*) and one simple sequence repeat (SSR) marker (*Xgwm295*) cosegregated completely with the lutein esterification trait. Each of the Haruhikari//Sunco/Indis.82 lines exhibited a parental combination for these loci, i.e. low/zero ester content in combination with marker alleles from Haruhikari or high ester content in combination with marker alleles from Sunco/Indis.82. Linkage mapping with both DArT and SSR marker data resulted in a genetic map (Fig. 2) of a 45.1-cM linkage group on which *Lute* and the markers *XwPt-1163*, *XwPt-3727* and *Xgwm295* map between the SSR markers *Xwmc438* (15.1 cM distal to *Lute*) and *Xbarc154* (17.1 cM proximal to *Lute*).

Consistent with the results from the nullisomic-tetrasomic analysis that implicate chromosome 7D in the genetic control of lutein esterification, the markers *XwPt-1163*, *XwPt-3727*, *Xgwm295* and *Xbarc154* have all previously been physically assigned to the terminal deletion 7DS4-0.61-1.00 of chromosome 7D (Francki et al., 2009; Cereals Data Base [http://www.cerealsdb.uk.net/cerealgenomics/CerealsDB]; Wilkinson et al., 2012; Sourdille et al., 2004). Thus, it seems that the action of a gene at the *Lute* locus on the short arm of chromosome 7D is required for the esterification of free lutein in the grain of hexaploid wheat. Haruhikari may lack a functional allele at that locus.

In the starchy endosperm esterification of lutein occurs after maturity, well after the death of the endosperm cells and when the grain moisture content is below 12%. Therefore, a gene that confers esterification would need to be transcribed and translated during grain development. Its protein product would need to be sequestered in an active or activatable form in close proximity to sources of its substrates: free lutein and the fatty acids palmitic acid and/or linoleic

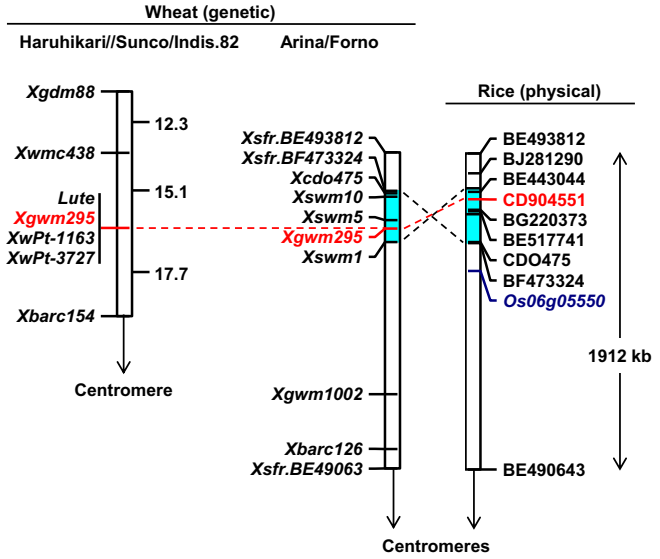

Fig. 2. Genomic locations of *Lute* in wheat and *LOC_Os06g05550* in rice. Left: A genetic map of a linkage group on the short arm of chromosome 6D of wheat, as mapped in a Haruhikari//Sunco/Indis.82 population (left side of figure), showing the lutein esterification locus *Lute* collocated with the SSR marker *Xgwm295*. Interval lengths on this map are shown in cM. Centre: Part of the short arm of chromosome 6D of wheat, as previously mapped in an Arina/Forno population [34]. This map is shown at the same scale as the Haruhikari//Sunco//Indis.82 map and the two maps are aligned based on the position of the common marker *Xgwm295*. Right: A physical map of part of the short arm of chromosome 6 of rice, as previously anchored [34] to the Arino/Forno map using expressed sequence tags [GenBank: BE493812, GenBank: BJ281290, GenBank: BE443044, GenBank: CD904551, GenBank: BG220373, GenBank: BE516641; GenBank: CDO475, GenBank: BG473324] with the addition of the position of a predicted gene, *LOC_Os06g05550*, which is annotated as encoding a GDSL lipase.

acid. Since free lutein is present in the membranes of the plastids, plastid membrane phospholipids, which have a fatty acid composition similar to lutein esters (Finnie et al., 2010), provide a likely source of fatty acids. We therefore hypothesise that the *Lute* locus contains a gene encoding a lipase that can act in reverse or as a transesterase. Given that water is a product of reverse reactions of lipases, the removal of water during storage of grain under low relative humidity may help drive a lipase-catalysed reaction towards ester synthesis. In chrysanthemum, a member of the GDSL lipase family is active in the esterification of pyrethrin (Kikuta et al., 2012). Similarly, it has been reported (Hamada et al., 2012) that an esterase with a GDSL motif could both hydrolyse or synthesise important ester compounds in rice bran. In earlier work on ester formation in bread wheat (Ahmad et al., 2013) we demonstrated that the constituent involved in esterification is very thermostable. In this context, it is worth noting that a thermostable (phospho) lipase has been reported to be present in rice bran (Bhardwaj et al., 2001).

The marker *Xgwm295*, which co-segregates with *Lute*, has previously been assigned to a wheat BAC (TaBAC470M18), which was in turn anchored to a position on rice chromosome 6 (Bossolini et al., 2006) based on similarity of sequences within the BAC to the gene *LOC_Os06g05000* on rice chromosome 6. That gene lies within a region in which there is an apparent inversion between wheat and rice (Fig. 2). About 310 kb proximal to *LOC_Os06g05000*, there is a putative gene, *LOC_Os06g05550*, that has been annotated as a GDSL-like lipase and found to be expressed in developing inflorescences and seeds (Rice Genome Annotation Project [http://rice.plantbiology.msu.edu]). Use of the predicted amino acid sequence (Genbank: AAP22477.1) of *LOC_Os06g05550* as a tBLASTx query against the UK454 Chinese Spring genomic sequence reads (Cereals Data Base [http://www.cerealsdb.uk.net/cerealgenomics/CerealsDB]; Wilkinson et al., 2012) returned Contig03415 as the best hit (E-value 4e-61, 57% identity, 66% coverage).

Among primers designed based on Contig03415 (Supplementary file 6), four pairs were selected that amplified products of the expected sizes from Chinese Spring but no products from two lines that are nullisomic for chromosome 7D (CS N7D-T7A and CS N7D-T7B). Two of these chromosome 7D-specific primer pairs (LF3/LR3 and LF4/LR4) amplified PCR products from genomic DNA of Chinese Spring and Sunco/Indis.82, but no products from Haruhikari. Two others (LF1/R1 and LE_F2/R2) amplified overlapping products from both Sunco/Indis.82 and Haruhikari. Sequencing and alignment of LF1/LR1 and LF2/LR2 amplicons revealed sequence polymorphisms between Sunco/Indis.82 and Haruhikari. Primers designed to flank two of the polymorphic regions (LF5/LR5 and LF6/LR6, Supplementary file 6) yielded codominant in HRM assays that detected polymorphism between Sunco/Indis.82 and Haruhikari (Supplementary file 7).

When the presence/absence polymorphisms were assayed on 117 doubled haploid lines, products of the expected sizes were amplified from every high-ester line and no products were amplified from any low/zero-ester line. Similarly, when the sequence polymorphisms were assayed with HRM, all high-ester lines gave the same melting curves as Sunco/Indis.82 and all low/zero-ester lines gave the same melting curves as Haruhikari (Supplementary file 7). This indicates genetic collocation of these polymorphisms with *Lute*. This positional information, in combination with the putative function of *LOC_Os06g05550*, make the wheat orthologue worthy of further investigation to determine whether it is expressed in endosperm tissue and whether it can be proven to be essential for lutein esterification.

4. Conclusions

The results presented here demonstrate that lutein mono- and di-esters can make up high proportions of the lutein in the endosperm and seed coat (but not embryo) of wheat. Lutein esterification is under the genetic control of the triploid endosperm and the capacity for esterification in wheat endosperm was mapped to a single locus (designated *Lute*) on the short arm of chromosome 7D. Polymorphisms in a wheat sequence with similarity to a putative GDSL-like lipase gene in rice were found to genetically collocate with *Lute*.

With the current focus on increasing lutein content in foods, issues relating to stability of this pigment in grain storage, transport and processing have become important. Because esterification seems to increase the stability of lutein, a better understanding of ester formation is critical to optimising the retention of this micronutrient through the food chain. Potentially there is also an opportunity to improve lutein stability in durum wheat via introgression of the esterification mechanism into that species.

Conflicts of interest

The authors declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jcs.2015.05.007.

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