

RESEARCH PAPER

# Identification of a major QTL controlling the content of B-type starch granules in *Aegilops*

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## Abstract

Starch within the endosperm of most species of the Triticeae has a unique bimodal granule morphology comprising large lenticular A-type granules and smaller near-spherical B-type granules. However, a few wild wheat species (*Aegilops*) are known to lack B-granules. *Ae. peregrina* and a synthetic tetraploid *Aegilops* with the same genome composition (SU) were found to differ in B-granule number. The synthetic tetraploid had normal A- and B-type starch granules whilst *Ae. peregrina* had only A-granules because the B-granules failed to initiate. A population segregating for B-granule number was generated by crossing these two accessions and was used to study the genetic basis of B-granule initiation. A combination of Bulk Segregant Analysis and QTL mapping identified a major QTL located on the short arm of chromosome 4S that accounted for 44.4% of the phenotypic variation. The lack of B-granules in polyploid *Aegilops* with diverse genomes suggests that the B-granule locus has been lost several times independently during the evolution of the Triticeae. It is proposed that the B-granule locus is susceptible to silencing during polyploidization and a model is presented to explain the observed data based on the assumption that the initiation of B-granules is controlled by a single major locus per haploid genome.

**Key words:** *Aegilops*, B-type granules, granule-size distribution, polyploidization, starch-granule initiation, wheat.

## Introduction

Whilst much is known about the synthesis of starch polymers in plants, little is known about starch-granule initiation or the determination of granule size and shape. Starch granules vary in size and shape between plant species showing that granule morphology is determined primarily by genetic factors. Members of the Triticeae grass family, which includes the economically important cereals wheat, barley, and rye, as well as wild grasses such as *Aegilops*, have, in their endosperm, starch with unique bimodal granule morphology (Evers 1971; Parker, 1985; Jane *et al.*, 1994). Each plastid in the endosperm of these species contains a single, lenticular A-type granule and several near-spherical B-type granules. In wheat, the A-type granules are 20–30 µm in diameter and the B-type granules are 2–7 µm in

diameter. The B-type granules form within wheat endosperm approximately 10 d later than the A-type granules (Parker, 1985; Bechtel *et al.*, 1990) and at least some of them are found within stroma-filled tubules (stromules) emanating from the plastids (Parker, 1985; Langeveld *et al.*, 2000). By contrast, the single A-type granule is confined to the main body of the plastid. Thus, the A- and B-type granules found in the Triticeae endosperm are the products of two granule-initiation events that are separated in time and space.

In contrast to the variation in starch granule-size distribution between species, very little genetic variation is observed between cultivars of domesticated wheat (Stoddard, 1999) and barley (Oliveira *et al.*, 1994). However, the small variations that do

exist have been shown to influence processing in both food and non-food industries. The small B-granules have both positive and negative impacts on industrial use. They do not precipitate during some wet processing procedures and are lost in the waste stream thereby decreasing yield and increasing the cost of managing waste treatment (Stoddard and Sarker, 2000). In wheat, B-granules negatively affect flour processing and bread-making quality (Park *et al.*, 2009), but positively affect pasta making (Soh *et al.*, 2006). This is thought to be due, at least in part, to the swelling capacity of B-granules: they can bind more water than A-type granules (Chiotelli *et al.*, 2002). In barley, it has been suggested that B-granules negatively affect beer making (Bathgate *et al.*, 1974). Half of the B-type granules are degraded during malting representing a 5% loss in potential alcohol production (Tillett and Bryce, 1993). Furthermore, the small B-type granules remaining after malting only partially gelatinize during mashing whilst the large A-type granules fully gelatinize. The partially-gelatinized B-granules make the wort very viscous and therefore difficult to filter and contribute to an undesirable haze in the final product.

Despite the lack of variation in starch-granule-size distribution within domesticated wheat and barley, a few species of *Aegilops* are known to have normal A-type granules, but are lacking or have reduced numbers of B-type granules (Stoddard and Sarker, 2000). This suggests that there are genes in the Triticeae which specifically control the formation of B-type granules and which are not required for A-granule synthesis. As none of the five genotypes of *Aegilops* lacking B-granules that were identified by Stoddard and Sarker (2000) has the same genome composition as any *Aegilops* with a normal B-granule number, the identification of the gene or genes responsible for B-granule initiation using traditional mapping approaches were precluded. To circumvent this, synthetic lines were examined to identify material with a different granule size-distribution to the natural species but with an equivalent genome composition. A cross between a synthetic and a natural *Aegilops* with varying granule-size distributions provided an opportunity to investigate the genetic basis of B-granule initiation.

## Materials and methods

### Plant material

*Aegilops* accessions (*Ae. crassa* four accessions 2240001–2240004, *Ae. peregrina* seven accessions 2070001–2070007, *Ae. tauschii* accession 2230001), and the *Aegilops*/wheat addition lines were supplied from the John Innes Centre germplasm collection, Norwich, UK by Dr Steve Reader. The synthetic *Aegilops* KU37 and KU41 were supplied from the Plant Germplasm Institute, Faculty of Agriculture, Kyoto University, Japan by Dr Sadao Sakamoto.

Grains were germinated on filter paper, in the dark at 17 °C and transferred to cereal mix [John Innes no.2, 30% grit, pH to 7.5 with lime and containing Exemptor (thiacloprid) for aphid control]. Seedlings were allowed to establish for 1–2 d and then vernalized for 6–8 weeks at 6/8 °C for a 16/8 h day/night. F<sub>2</sub> plants for segregation analysis were grown in the John Innes Centre field plots in 2008. No fertilizer or pesticides/fungicides were applied.

All other plants were grown in individual pots in cereal mix in a greenhouse at a minimum temperature of 12 °C or in a controlled-environment room at a constant temperature of 15 °C, with 16/8 h light/dark and 70% humidity.

### Microscopic determination of the B-granule phenotype

To assess the B-granule phenotype qualitatively, individual seeds were cut to reveal the endosperm and the cut surface was scratched to remove a sample of starch to a glass slide. Lugol's solution (Sigma–Aldrich, UK) was added to stain the starch which was then observed under a light microscope. The presence or absence of large numbers of small B-type granules was noted.

For quantitative measurement of the proportion of small granules, one-quarter to one-third of a seed was removed and ground in 0.5 ml of water in a 4 ml tube containing a ball bearing (9 mm diameter) using a Geno/Grinder 2000 (SPEX CertiPrep Ltd, UK) at 1500 strokes min<sup>-1</sup> for 20 s. 15 µl of the extract was placed on a glass slide together with 5 µl of Lugol's solution (Sigma–Aldrich, UK) and the slides were observed under a light microscope (×10 objective). A minimum of three images from different areas of each slide were taken. These micrographs were analysed using the count/size algorithm incorporated within the Image ProPlus image analysis software ([www.MediaCy.com](http://www.MediaCy.com)) which was calibrated against the microscope objective. Granules were assessed as dark objects against a light background and size and roundness filters were further used to prevent counting of non-starch material. For size-distribution measurements, clean image borders were used and touching objects were split using the watershed split and manual split functions. The data were divided into two classes, granules >10 µm or <10 µm diameter and the percentage in each class returned. The data for a minimum of three aliquots of each extract were used to provide a value for a single grain.

### Quantification of the number of granules per endosperm

The methods used in Burton *et al.* (2002) for counting granules in developing barley endosperm were adapted for use with developing *Aegilops*. Endosperm dissected from an individual developing grain was homogenized in 500 µl extraction medium (50 mM HEPES pH 7.8, 10 mM EDTA, 10 mM DTT, and 0.1 mg ml<sup>-1</sup> proteinase K) using a Genogrinder, (1500 rpm, 20 s). The homogenate was incubated at 37 °C for 1–1.5 h, centrifuged at 28 000 g for 10 min and the supernatant discarded. The pellet was washed successively in 1 ml aliquots of 20 g l<sup>-1</sup> SDS (once) and water (twice) and the resulting starch preparation was resuspended in 120–500 µl water (dependent on the size of the endosperm). Aliquots (50 µl) of the suspension were removed and combined with 100 µl iodine and 850 µl water. The number of granules ml<sup>-1</sup> was estimated using a haemocytometer slide with a unit volume of 0.00625 mm<sup>3</sup>. For each single-grain extract, two separate dilutions were made and each was examined on an individual slide. The number of granules per unit volume was determined from five images per slide using Image ProPlus software as described above, with the exception that for counting data, clean borders were applied to two sides of the image only. Data for granule-size distribution was also recorded by re-analysing the images as described above. The data were used to calculate the number of granules per endosperm, and the number and % of small granules.

### DNA preparation

Genomic DNA was extracted from 50 mg of leaf tissue using the Qiagen DNeasy 96 Plant Kit, according to the manufacturer's instructions except that a Genogrinder was used instead of a TissueLyser for grinding samples. Samples were ground in the Genogrinder at 1500 strokes min<sup>-1</sup> for 1.5 min. This procedure yielded 1–15 µg of DNA, at a concentration of 5–60 µg µl<sup>-1</sup>.

The DNA concentration was adjusted to 4 ng  $\mu\text{l}^{-1}$  and the DNA samples were stored at  $-20\text{ }^{\circ}\text{C}$ .

### Genotyping

Markers were chosen from two publically-available COS marker collections, the Wheat Genetic Improvement Network (WGIN) (<http://www.wgin.org.uk/resources/Markers/TAMarkers.php>) and Tools and Resources (TR) collections (<http://www.modelcrop.org/cgi-bin/gbrowse/brachyv1/>). The labelled primers for these markers are available upon request, whilst stocks last from Dr S Griffiths, John Innes Centre. All markers were designed to produce PCR products ranging from 100–600 bp in length. Reverse PCR primers were directly labelled with a fluorescent dye (6-FAM).

Most of the markers were analysed as follows. PCR products were amplified from 10 ng of genomic DNA in 6.25  $\mu\text{l}$  reactions containing 3.125  $\mu\text{l}$  Qiagen HotStarTaq Master Mix and 2  $\mu\text{M}$  each of the forward and reverse primers. PCR amplifications were performed on a Peltier Thermal cycler using the following programmes. For WGIN markers: 95  $^{\circ}\text{C}$  (15 min), 39 cycles of [95  $^{\circ}\text{C}$  (0.5 min), 58  $^{\circ}\text{C}$  (0.5 min), 72  $^{\circ}\text{C}$  (0.5 min)], hold at 72  $^{\circ}\text{C}$  (5 min) then at 10  $^{\circ}\text{C}$ . For the TR primers: 94  $^{\circ}\text{C}$  (10 min), 16 cycles of [95  $^{\circ}\text{C}$  (0.5 min), 58  $^{\circ}\text{C}$  (1 min), decreasing by 0.5  $^{\circ}\text{C}$  per cycle to 50  $^{\circ}\text{C}$ , 72  $^{\circ}\text{C}$  (1 min)], 25 cycles of [94  $^{\circ}\text{C}$  (0.5 min), 50  $^{\circ}\text{C}$  (1 min), 72  $^{\circ}\text{C}$  (1 min)], hold at 15  $^{\circ}\text{C}$ .

The PCR products were prepared for analysis as follows. The PCR reaction was diluted 30-fold in sterile distilled water and then 1  $\mu\text{l}$  was added to 9  $\mu\text{l}$  of a mixture of GeneScan™ 500 LIZ™ Size Standard (Applied Biosystems, USA) and highly deionized (Hi-Di)™ (Applied Biosystems, USA) formamide mixture. The mixture was prepared by adding 5  $\mu\text{l}$  of GeneScan™ 500 LIZ™ Size Standard to 1 ml Hi-Di™ formamide. All samples were submitted to the John Innes Genome Centre for genotyping using a POP7 column attached to a 3730xl® DNA Analyzer (Applied Biosystems, USA). The results were analysed using GeneMapper v4.0.

A minority of the markers were also analysed by single strand conformation polymorphism (SSCP) essentially according to Martins-Lopes *et al.* (2001). The PCR products were amplified in 15  $\mu\text{l}$  reactions containing 5  $\mu\text{l}$  of DNA (10 ng  $\mu\text{l}^{-1}$ ), 1.5  $\mu\text{l}$  of Forward and Reverse primer (2  $\mu\text{M}$ ), 0.07  $\mu\text{l}$  Taq polymerase (Roche), 1.5  $\mu\text{l}$  of PCR buffer (supplied with the polymerase, containing  $\text{MgCl}_2$ ), 1.5  $\mu\text{l}$  of dNTP (25 mM) and water. The PCR products were denatured and separated on a vertical MDE™ non-denaturing gel made with mutation detection enhancement (MDE) gel solution (16.5 ml; BMA, Rockland ME, USA), TTE buffer (2 ml; Severn Biotech Ltd., Kidderminster, Worcs., UK), 50% glycerol (12 ml), and 35 ml of water, and polymerized with 300  $\mu\text{l}$  of 10% APS and 30  $\mu\text{l}$  of TEMED. Following electrophoresis at 4 W and 5  $^{\circ}\text{C}$ , the products were visualized with silver stain as described by Bassam *et al.* (1991).

Four polymorphic markers (TR126, TR132, TR119, 4G) were identified and scored in Chinese Spring-*Ae. peregrina* addition lines (Friebe *et al.*, 1996) containing the following chromosomes (or chromosome arms) from *Ae. peregrina*: 4U, 4U (short arm), 4U (long arm), 4S, 4S (short arm), 4S (long arm).

### Construction of genetic map and QTL analysis

Linkage analysis and map construction was carried out using JoinMap® version 3.0 (Van Ooijen, 2006). Linkage groups were determined using a Divergent log-of-odds (LOD) threshold of 4.0 and genetic distances were computed using the Kosambi regression. Segregation ratios of markers were determined and statistically tested for deviations using the chi-square test. QTL were detected by Composite Interval Mapping as implemented in Windows QTL Cartographer (Wang *et al.*, 2004), Model 6, with five markers, 1 cM walking speed and a 10 cM window. A LOD threshold of 3.0 was used which is above the value of 2.8 calculated from 1000 permutations using a significance level of 0.01.

### Statistical analysis

Analysis of variance for the markers defining the QTL peak (TR129 and 4G) was performed using GenStat 11th edition (Payne *et al.*, 2008) and orthogonal contrasts (linear and quadratic components) were used to determine the degree of dominance of these markers.

## Results

### Analysis of starch-granule-size distribution in *Aegilops* species

In a previous study, Stoddard and Sarker (2000) found five *Aegilops* species that lacked or had low numbers of small, B-type starch granules. Accessions from these and additional *Aegilops* species were obtained from the John Innes Centre germplasm collection and assessed by microscopic examination of the endosperm starch for the abundance of small spherical granules (B-type and small A-type) relative to the large lenticular granules (A-type). All of the *Ae. crassa* and *Ae. peregrina* accessions examined had relatively few small granules (data not shown) and the results were therefore in agreement with those of Stoddard and Sarker (2000). The genome composition and granule morphology of these accessions are shown in Table 1 together with those of a selection of other species and accessions with normal B-granule content.

### Synthetic SU tetraploids possess B-granules

The natural tetraploids *Ae. peregrina* and *Ae. kotschyi* both have few, if any, B-granules (Table 1) and both have the same two genomes (S and U). To determine whether polyploidization between the S and U genomes is always associated with the lack of B-granules, starch from the seeds of two synthetic SU tetraploids, KU37 and KU41, was examined. KU37 was derived from a cross between the diploids *Ae. sharonensis* (S<sup>sh</sup>) and *Ae. umbellulata* (U) and KU41 from a cross between the diploids *Ae. bicornis* (S<sup>b</sup>) and *Ae. umbellulata* (U) (Tanaka, 1955, 1983). Unlike the natural SU tetraploids, both synthetics were found to have B-type granules (Fig. 1).

### B-granule initiation fails to occur in *Ae. peregrina*

The absence of the smaller B-type granules in *Ae. peregrina* could be due to several factors. For example, B-type granules might initiate normally in these species but then continue to grow to form additional A-granules. Alternatively, B-granules could fail to initiate leading to the presence of A-granules only. To investigate this, the timing and extent of granule initiation during grain development in the natural (*Ae. peregrina*) and synthetic (KU37) tetraploids with S and U genomes were compared (Fig. 2).

A time-course of grain development (Fig. 2A, B) and granule initiation (Fig. 2C, D) was conducted on plants grown together in a controlled environment room to minimize the environmental effects on granule size and

**Table 1.** Starch granule morphology and genome composition of selected *Aegilops* species

The genome composition of five *Aegilops* that lacked or had low numbers of small, B-type starch granules (Stoddard and Sarker, 2000) are shown together with those of a selection of other species and accessions with normal B-granule content.

Granule morphology	Species <sup>c</sup>	Synonyms <sup>d</sup>	Genome symbols <sup>e</sup>
A-type only (no B-type)	<i>Ae. peregrina</i> <sup>a, b</sup>	<i>Ae. variabilis</i>	SU
	<i>T. peregrinum</i>		
	<i>Ae. kotschy</i> <sup>a</sup>	<i>T. kotschy</i>	US
	<i>Ae. crassa</i> <sup>a, b</sup>	<i>T. crassum</i>	DM
	<i>Ae. crassa</i> <sup>a</sup>	<i>T. crassum</i>	DDM
A- and B-type	<i>Ae. juvenalis</i> <sup>a</sup>	<i>T. juvenale</i>	DMU
	Synthetic KU37 <sup>b</sup>	–	US <sup>sh</sup>
	Synthetic KU41 <sup>b</sup>	–	US <sup>b</sup>
	<i>Ae. umbellulata</i> <sup>a</sup>	<i>T. umbellulatum</i>	U
	<i>Ae. comosa</i> <sup>a</sup>	<i>T. comosum</i>	M
	<i>Ae. longissima</i> <sup>a</sup>	<i>T. longissimum</i>	S <sup>l</sup>
	<i>Ae. sharonensis</i> <sup>a</sup>	<i>Ae. longissima</i> var. <i>sharonensis</i>	S <sup>sh</sup>
		<i>T. sharonense</i>	
	<i>Ae. bicornis</i> <sup>a</sup>	<i>T. bicorne</i>	S <sup>b</sup>
	<i>Ae. tauschii</i> <sup>b</sup>	<i>Ae. squarrosa</i> <i>T. tauschii</i>	D

<sup>a</sup> Granule morphology according to Stoddard and Sarker (2000).

<sup>b</sup> Granule morphology according to this study.

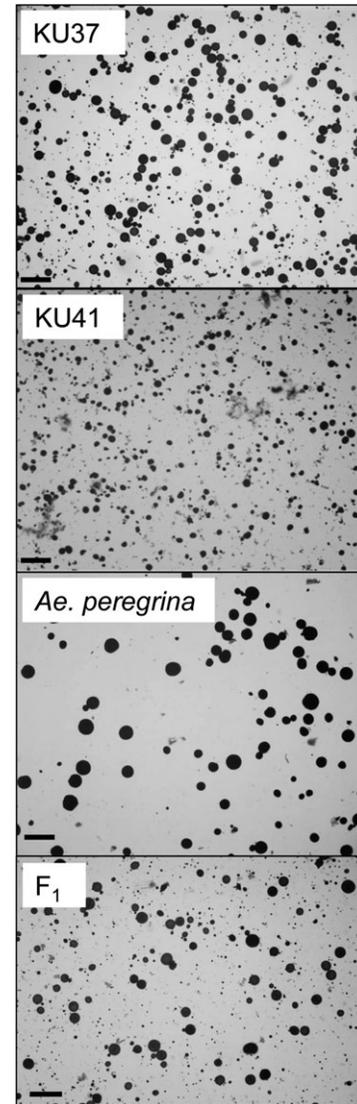
<sup>c</sup> Nomenclature follows van Slageran (1994).

<sup>d</sup> Synonyms follows Kimber and Feldman (1987).

<sup>e</sup> Genome symbols follow the Wheat Classification System ([www.k-state.edu/wgrc/Taxonomy](http://www.k-state.edu/wgrc/Taxonomy)).

number. The results showed that, in KU37, the number of granules per endosperm remains constant from 4 days after anthesis (DAA) until approximately 19 DAA. The number of starch granules within the endosperm then doubles during the final 2 weeks of grain filling. There are therefore two waves of granule initiation in KU37, as in most other Triticeae species. In *Ae. Peregrina*, however, the number of granules per endosperm remains constant from 4 DAA until maturity.

The granule-size distribution throughout development was also recorded (Fig. 2D). These data show that the proportion of small granules (<10 µm in diameter) in *Ae. peregrina* decreased steadily throughout development. At maturity, there were still some granules in *Ae. peregrina* that had not grown larger than 10 µm in diameter. This suggests that granules that were initiated before 4 DAA grew continuously until maturity (Fig. 2D). In KU37, the proportion of small granules also steadily decreased prior to the initiation of the B-granules at approximately 19 DAA. At this point, the increase in the number of granules observed in the endosperm (Fig. 2B) caused the proportion of small granules in the endosperm to remain constant throughout the rest of development. The small granules present in mature *Ae. peregrina* grains are, therefore, small A-type granules whilst those in KU37 are a mixture of small A-type and the newly-formed B-type

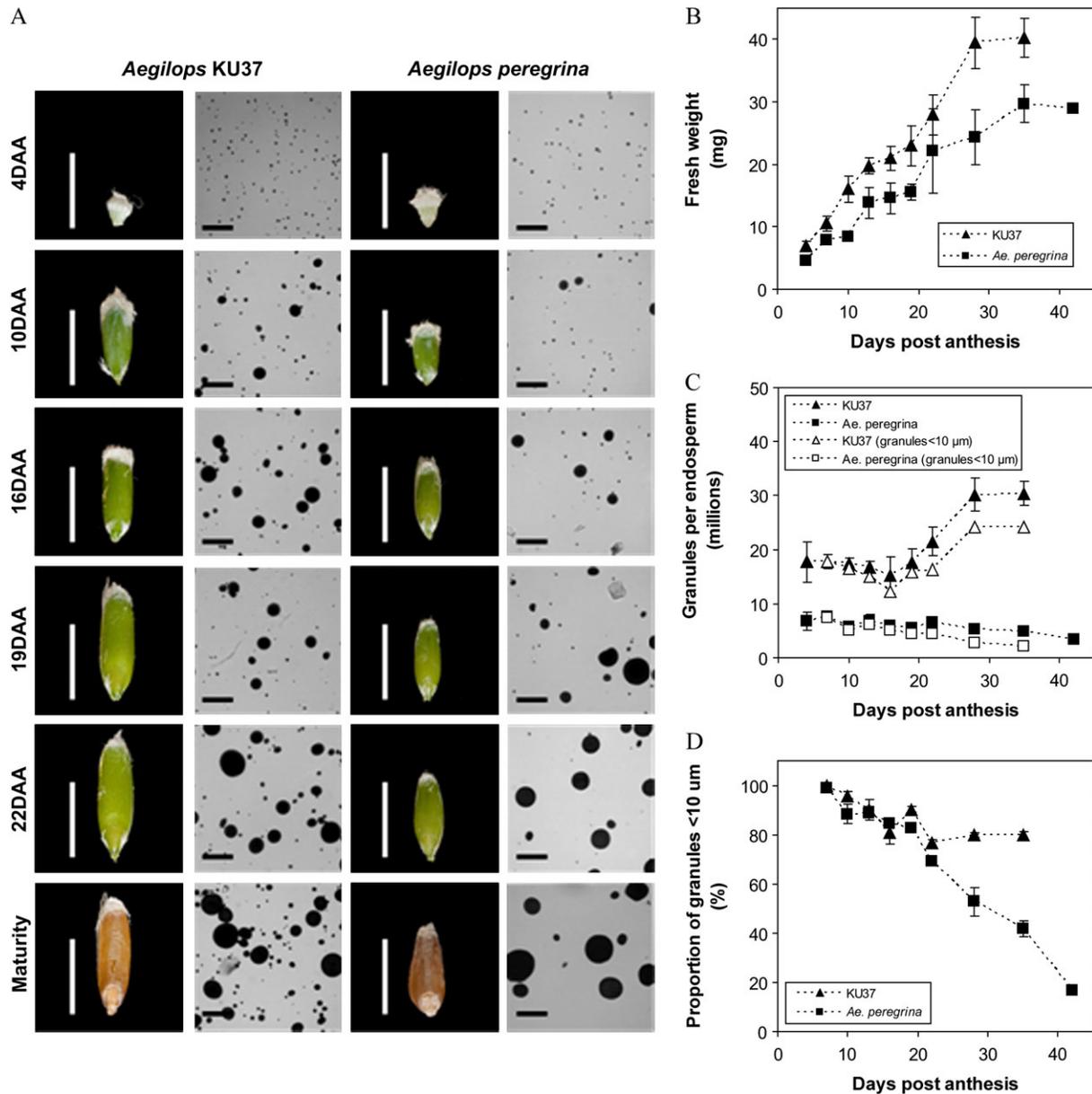


**Fig. 1.** Starch granule morphology. Light micrographs of iodine-stained starch from mature endosperms of the synthetic tetraploids KU37 and KU41, the natural tetraploid *Ae. peregrina* and a typical F<sub>1</sub> seed from a cross between KU37 (female) and *Ae. peregrina* (pollen donor). The scale bar is 100 µm.

granules. These data suggest that, in *Ae. peregrina*, the A-type granules usually initiate early in development but that the second wave of initiation that would normally give rise to B-type granules fails to occur.

#### *The B-granule-less trait segregates in a tetraploid F<sub>2</sub> population*

The phenotypic variation within the SU tetraploids provided an opportunity to investigate the genetic basis of B-granule initiation. *Ae. peregrina* was hybridized with both KU37 and KU41. *Ae. peregrina* was used as both the male and the female parent, but only the crosses with *Ae. peregrina* as the male parent were successful. The F<sub>1</sub> seed produced was



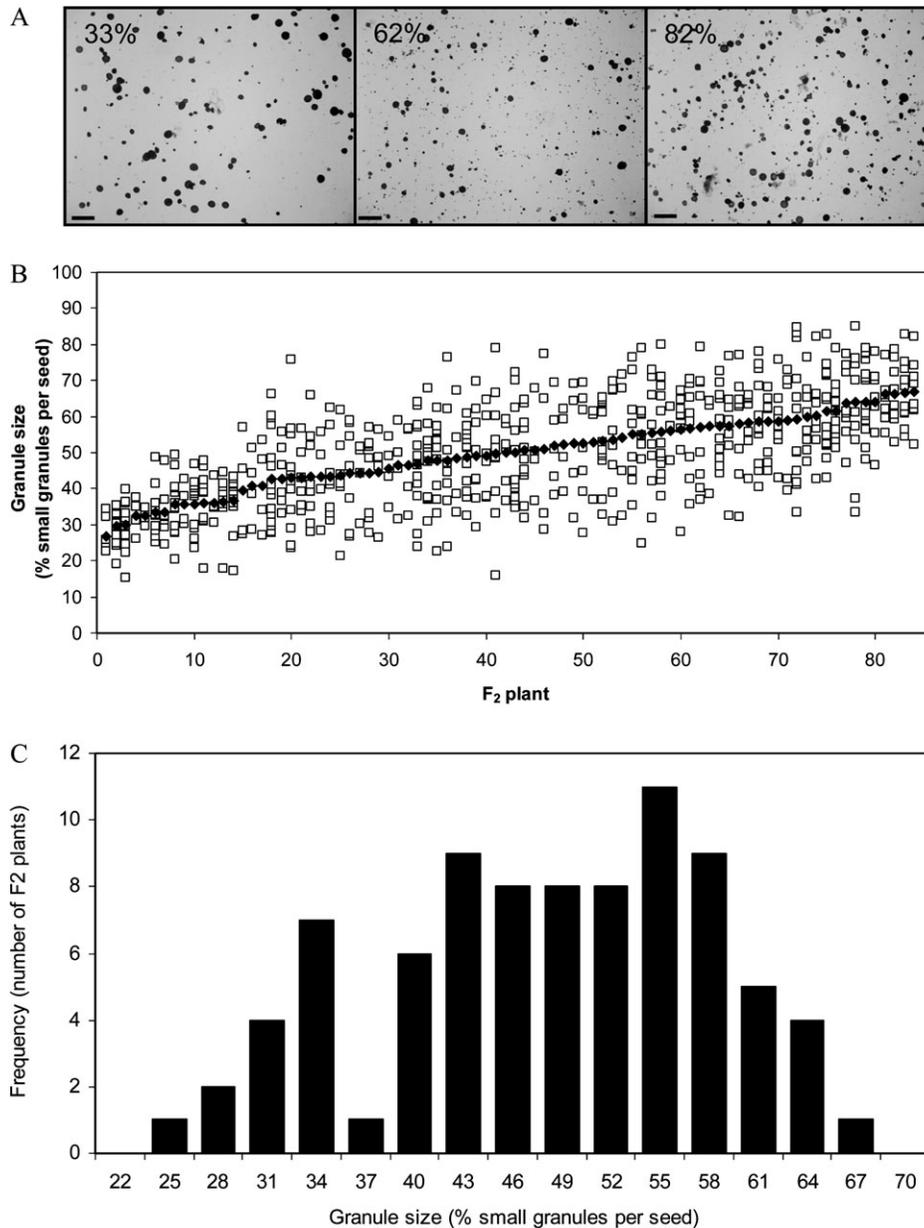
**Fig. 2.** Starch granule number during grain development. Grains were harvested at various stages of development from 4 days after anthesis (DAA) through to maturity. (A) Images of developing grains (scale bar 5 mm) and iodine-stained starch (scale bar 50 μm) from *KU37* (left) and *Ae. peregrina* (right) from 4 DAA to maturity. (B) Fresh weight, (C) number of starch granules, and (D) percentage of granules less than 10 μm diameter were also recorded. Values are the means  $\pm$ SE of measurements on a minimum of three separate grains.

sown and the resulting plants were allowed to self-fertilize. Viable  $F_2$  seed was produced by the *KU37* hybrid but not by the *KU41* hybrid. Evidence that self-fertilization of *KU37* had not taken place came from an examination of the  $F_2$  plants. The phenotypes of the  $F_2$  plants were varied and showed characteristics inherited from *Ae. peregrina* as well as from *KU37* (see Supplementary Fig. S1 at *JXB* online).

To test whether the lack of B-granules in *Ae. peregrina* was recessive or dominant, the phenotype of the  $F_1$  seed produced from crosses between the two synthetics (*KU37* and *KU42*) with *Ae. peregrina* was determined. All of the  $F_1$  progeny from both the *KU37* (Fig. 1) and *KU41* (data not

shown) crosses had B-type granules. This suggested that the lack of B-granules in *Ae. peregrina* is recessive.

To investigate the pattern of inheritance of the B-granule-less trait, the segregation of B-granule number was examined in the progeny from the *Ae. peregrina*  $\times$  *KU37* cross. Ninety-three  $F_2$  seeds from eight different  $F_1$  plants were sown and the  $F_3$  seeds from individual  $F_2$  plants were collected and phenotyped (Fig. 3). A preliminary qualitative inspection of a small sub-set of the  $F_3$  seeds suggested that the  $F_2$  population was segregating for the B-granule-less trait. Seeds like those of the parents, with high or low numbers of B-granules, were observed as well as seeds with intermediate numbers of B-granules (Fig. 3A).



**Fig. 3.** Analysis of granule-size distribution. The  $F_3$  seeds were harvested from individual  $F_2$  plants. Starch granules were extracted from the endosperm of 4–16 individual  $F_3$  seeds per plant. The proportion of small-granules (10  $\mu\text{m}$  or less in diameter) in the endosperm was examined by microscopy and quantified by image analysis. (A) Examples of micrographs of seeds with low, medium, and high numbers of small granules. The percentage of small granules is indicated. The scale bar is 100  $\mu\text{m}$ . (B) The percentage of small granules in individual  $F_3$  seeds from 84 separate  $F_2$  plants. Open squares are values for individual seeds and closed diamonds are means for individual  $F_2$  plants. (C) The frequency of  $F_2$  plants per granule size category. Mean granules sizes for individual  $F_2$  plants are plotted against granule-size bins of three units.

To analyse the phenotypic distribution in greater depth, the percentage of small granules (with diameters of 10  $\mu\text{m}$  or less) in 4–16  $F_3$  seeds from each  $F_2$  plant was quantified using image-analysis of light micrographs such as those in Fig. 3A. The small granule category in this analysis does not distinguish between small A-granules which failed to grow and B-granules. Of the 93  $F_2$  plants sown, 84 produced seed which were phenotyped. In agreement with the preliminary qualitative study, granule quantification showed that the proportion of small-granules varied

amongst the seeds of the  $F_2$  plants (Fig. 3B) ranging from less than 30% small granules to over 65%. The variation observed was near-continuous with slight discontinuities in the distribution at either end of the range. The frequency distribution of  $F_2$  plants with different mean granule sizes (Fig. 3C) was near-normal but there was a suggestion of a separation into two groups: 14 plants had <37% small granules and 70 plants had >37% small granules. Qualitative examination of the seeds of the 14  $F_2$  plants with low numbers of small granules showed that their starch

granule-size-distributions were indistinguishable from that of the *Ae. peregrina* parent. The 14:70 ratio is not statistically significantly different from the 1:3 distribution expected for the segregation of a single recessive gene ( $\chi^2$  test  $P=0.078$ ). However, other more complex gene models cannot be ruled out. Overall, these data suggest that the inheritance of granule-size distribution can be treated as a quantitative trait but that the phenotypic distribution in the  $F_2$  could also be explained by a poorly-resolved Mendelian trait, possibly indicating segregation at a single locus.

#### Genes associated with B-granule content are located on the short arm of chromosome 4S

Bulked Segregant Analysis was used to identify molecular markers associated with loci that influence B-granule number. DNAs from 30  $F_3$  seeds which differed in B-granule content (15 with normal numbers of B-granules and 15 lacking B-granules) were pooled into two bulks. In each bulk, the individuals will be enriched in the particular genomic regions responsible for the B-granule trait but will be segregating in other regions.

First, to identify suitable markers, a total of 197 markers designed from wheat or rice DNA sequences [comprising 74 single-sequence-repeat (SSR) and 123 conserved-orthologous-sequence (COS) markers] were tested on the parental DNAs. As the S and U genomes of *Aegilops* are known to be most similar to the B (Sarkar and Stebbins, 1956) and D (Zhang *et al.*, 1998) genomes of wheat, respectively, the SSR markers used were randomly selected from these wheat genomes. Of the primers tested, 194 successfully amplified a product from the *Aegilops* genome of which 85 (32 SSR and 53 COS markers) were polymorphic between the parents. This confirms that wheat SSR markers can be used successfully to identify polymorphisms

in *Aegilops*, as was shown previously (Adonina *et al.*, 2005; Bandopadhyay *et al.*, 2004) and it also shows that rice COS markers can be used to amplify homologues in other grasses.

The 85 polymorphic markers (COS and SSR) were tested on the two bulked DNA samples. These markers were distributed randomly throughout the genome and included each wheat homoeologous chromosome group. Four markers were found to be polymorphic between the bulk with normal B-granule content and the bulk lacking B-granules. These were the COS markers TR119, TR129, 4G, and the microsatellite marker *Psp3078* (Bryan *et al.*, 1997; Stephenson *et al.*, 1998). Markers 4G and *Psp3078* have been mapped to the group 4 chromosomes of wheat (Sorrells *et al.*, 2003; [http://wheat.pw.usda.gov/wEST/binmaps/wheat4\\_rice.html](http://wheat.pw.usda.gov/wEST/binmaps/wheat4_rice.html)). The other two markers were designed to genes on rice chromosome 3 (Table 2) which is known to be co-linear with group 4 chromosomes of wheat (The rice chromosome 3 sequencing consortium, 2005) and with barley 4H (bioinf.scri.ac.uk/strudel/). This suggests that a locus determining B-granule initiation is located in a region of the *Aegilops* genome corresponding to the Triticeae group 4 chromosomes.

The *Aegilops* and wheat genomes are known to be highly co-linear, for example, wheat chromosome 4D is homoeologous to *Aegilops* 4S (Zhang *et al.*, 2001). Thus, it is likely that the markers that were found to be linked to the B-granule phenotype are on chromosome 4S or 4U in *Ae. peregrina*. To test this idea, these markers and others also predicted to be on the group 4 chromosomes of wheat (based on co-linearity with rice and *Brachypodium*) were tested for polymorphism between wheat cv. 'Chinese Spring' and *Ae. peregrina* (Table 2). Four polymorphic markers (TR126, TR132, TR119, 4G) were identified and scored in Chinese Spring–*Ae. peregrina* addition lines (Friebe *et al.*, 1996). The analysis showed that the polymorphic bands from all four markers map to chromosome 4S. Markers TR126, TR132, and 4G

**Table 2.** COS and microsatellite markers used to genotype the *Aegilops*

The COS markers were designed to span one or more introns in a rice gene or were designed against a wheat transcript. All markers are available via [www.modelcrops.org](http://www.modelcrops.org). The *Brachypodium* homologs were identified by BLASTn using the wheat sequences ([www.brachybase.org](http://www.brachybase.org)).

Marker	Forward primer (5'-3')	Reverse primer (5'-3')	Source sequence	Wheat homologue (EST or EST assembly)	<i>B. distachyon</i> homologue (E value)
TR119 <sup>a, c</sup>	GCAGCAGGAATTCTACATCAG	TGACGTGGAAGAGGTTTCAC	Os03g52780	Ta32373_4565	Bradi1g68120 (1e <sup>-114</sup> )
TR129 <sup>a</sup>	TTTGTAAAGGCATCCAACATG	CTGAGTAGTACCGAAGCAC	Os03g49230	CD865921	Bradi1g11700 (5e <sup>-42</sup> )
<i>Psp3078</i> <sup>a</sup>	TTATTTTGACGCTTGACATA	TTAGGGCTCATAAGGGTCTA	Microsatellite	–	–
4G <sup>a, c</sup>	GCAATCACGAACGGCTCGATCA	ATCTGGCAGCTTGCCAAGGCT	BE442666	BE442666	Bradi1g11670 (1e <sup>-60</sup> )
TR113 <sup>b</sup>	TCCTACAATCAAGGCTCTGTG	TTCTCTGAAAGCCTCTCCAG	Os03g03470	CV768642	Bradi1g76790 (1e <sup>-51</sup> )
TR126 <sup>b, c</sup>	CCGGATGCTGTTGTATCCTG	TCATTCTTGGAGAACAGCGAG	Os03g42810	CA735797	Bradi1g07410 (1e <sup>-148</sup> ), Bradi5g24240 (1e <sup>-135</sup> )
TR132 <sup>b, c</sup>	GTTCCATTCCCTAAGAAGATC	GTCCTGGTCAAAGTGTGAG	Os03g50230	CD906287	Bradi4g00230 (3e <sup>-21</sup> )
TR133 <sup>b</sup>	CATTGAACAGCCTTGCCAATG	TCTGCCAGATACTGTTCCAGAC	Os03g51830	TA25917_4565	Bradi1g10000 (7e <sup>-81</sup> )
TR151 <sup>b</sup>	GGTATAAGCCAGAGGTTGGTG	AACCGCCAGCGCTTTGGAG	Os04g43922	TA38377_4565	Bradi1g04570 (1e <sup>-107</sup> )

<sup>a</sup> Markers used for Bulk Segregant Analysis.

<sup>b</sup> Additional markers used for linkage analysis.

<sup>c</sup> Markers shown by analysis of Chinese spring–*Ae. peregrina* addition lines to be located on chromosome 4S.

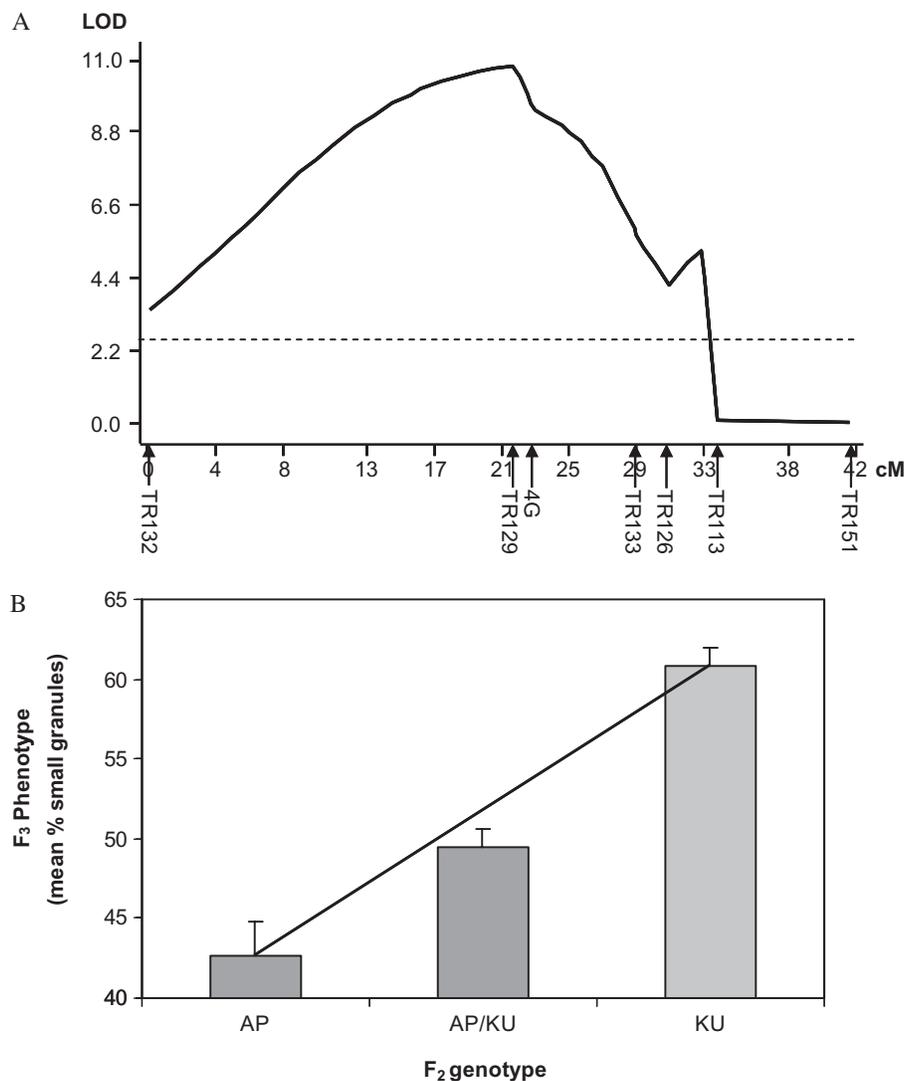
are located on the short arm of chromosome 4S and TR119 is located on the long arm of chromosome 4S. Thus, the markers that are linked to the B-granule phenotype are on *Aegilops* chromosome 4S.

*A major QTL for granule size is located on chromosome 4S*

To fine map the B-granule locus and to assess its quantitative contribution to the control of B-granule number, individuals from the *Ae. peregrina* x KU37 F<sub>2</sub> population were screened with the markers shown in Table 2. Linkage analysis showed that seven of the markers tested formed a linkage group (Fig. 4A). None of the markers used showed significant segregation distortion, i.e. equal proportions of parental alleles were observed for all

markers across the population. QTL analysis using the quantitative values for average granule size per F<sub>2</sub> plant (Fig. 3B) revealed a major QTL for granule size that was associated with this linkage group (Fig. 4A). This QTL explained 44.4% of the phenotypic variation of this trait within the population. Three of the markers within the linkage group had been mapped to the short arm of chromosome 4S, including TR132 and TR126 which flank the QTL peak. Thus, a major QTL for B-granule number is located on the short arm of chromosome 4S.

As expected, the *Ae. peregrina* allele at the QTL had a negative effect on the percentage of small granules (<10 µm diameter). To investigate whether the effect of the KU37 allele was additive or dominant, individuals were categorized according to their alleles at the two markers closest to the QTL peak (TR129, 4G) and the mean



**Fig. 4.** Genetic location of the B-granule QTL on the short arm of chromosome 4S. (A) Genotype data for the linked markers shown in Table 2 together with the granule-size phenotypes for 84 F<sub>2</sub> plants (average granule size per F<sub>2</sub> plant) shown in Fig. 3B were used to calculate the position of a QTL responsible for variation in B-granule number. (B) The F<sub>2</sub> plants were grouped according to the genotype of markers 4G and TR129 and the mean value of the associated phenotype was assessed. Group AP, both markers are homozygous for the *Ae. peregrina* genotype; KU, both markers are homozygous for the KU37 genotype; and AP/KU both marker are heterozygous. Means are all significantly different at  $P < 0.01$ . Values are means  $\pm$ SE of the percentage of small granules per seed.

phenotypic values were calculated (Fig. 4B). Individuals carrying the *Ae. peregrina* alleles at TR129 and 4G had an average of 42.7% small granules, whereas lines with the KU37 alleles had a statistically significantly higher average (60.8%,  $P < 0.0001$ ). Heterozygous individuals had an intermediate average of small granules (49.5%), which was significantly different from both homozygous classes ( $P < 0.01$ ). The linear and quadratic components of these markers were determined through contrasts to test the additive and dominant nature of the QTL. The linear (additive) contrast was highly-significant ( $P < 0.01$ ) whilst the quadratic (dominant) contrast was not significant ( $P = 0.18$ ) suggesting that the effect of the KU37 allele was additive.

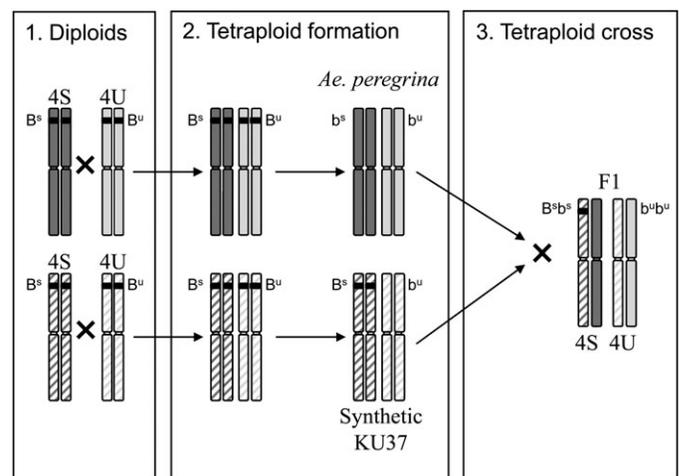
## Discussion

To understand the genetic basis of the B-granule trait, *Aegilops* lines with and without B-granules were identified and crossed to create an F<sub>2</sub> population that was segregating for B-granule content. Analysis of this population allowed the identification of a major QTL controlling B-granules within a linkage group of approximately 40 cM on the short arm of chromosome 4S. Prior to this work, fertile crosses between the *Aegilops* species with and without B-granules were not possible due to the lack of observed variation in granule morphology between sexually-compatible individuals. The key to this work was the identification of synthetic polyploid *Aegilops* lines with the same genome composition but different granule-size distribution from the natural species. The analysis of granule size and number in *Aegilops* during development showed that, as in other Triticeae species, the B-type granules initiate later than the A-type granules. In *Ae. peregrina*, the timing of A-granule initiation appears to be normal but the second wave of granule initiation fails to occur. Thus, the major B-granule locus on chromosome 4S is required for the initiation of B-granules but not for A-granules. This suggests that there are specific genes in the Triticeae that control the initiation of B-type granules without significantly affecting A-granule initiation.

All of the *Aegilops* species previously shown to lack B-type granules are polyploid whereas their diploid progenitors have normal granule-size distributions (Table 1; Stoddard and Sarker, 2000). However, not all *Aegilops* polyploids lack B-granules and those that do have a variety of different genome compositions. The association of the trait with polyploids, but not diploids, and its lack of association with particular genomes are characteristics of a trait controlled by genes that are silenced during polyploidization. A well-documented example of such a trait in wheat is grain hardness which is controlled by the *Ha* locus. The *Pina* and *Pinb* genes at the *Ha* locus are present in the diploid progenitors of durum wheat [*Triticum turgidum* (AB)] but were deleted from both the A and the B genomes during the tetraploidization event. Consequently, durum wheat is hard in texture. A survey of *pin* gene polymorphism amongst diploid and polyploid *Triticum* and

*Aegilops* showed that, although the *Ha* locus is absolutely conserved in diploid species, it has been deleted independently and recurrently at least four times in different polyploids (Li *et al.*, 2008). Accumulating evidence suggests that polyploidization is accompanied by extensive silencing of genes (genetic and epigenetic) that occurs within 2–3 generations and probably starts during or soon after F<sub>1</sub> zygote formation (Baum and Feldman, 2010; Feldman and Levy, 2009). For example, up to 14% of the sequences examined by DNA hybridization were lost in a cross between *Ae. sharonensis* (S<sup>sh</sup>) and *Ae. umbellulata* (U) (Shaked *et al.*, 2001). The genes affected vary from cross to cross as shown by studies of the *pin* genes in synthetic tetraploids created by crossing the diploid progenitors of *T. turgidum*. The *pin* genes showed rearrangements in approximately 1% of the synthetic tetraploids (Li *et al.*, 2008). These data show that (i) polyploidization can cause gene deletion, (ii) loss of both homoeologues during tetraploidization is possible, and (iii) most synthetic polyploids inherit the parental gene sequences.

It is suggested here that the loss of B-granules might have occurred during polyploidization in some *Aegilops* and the following model is proposed to explain the genetic basis of the B-type starch granule trait in the segregating population of *Aegilops* studied here (Fig. 5). The natural tetraploid *Ae. peregrina* (SU) was derived from diploid progenitors



**Fig. 5.** A model of the evolution of the B-granule-less trait in *Aegilops*. The diploid progenitors (panel 1) have seven chromosomes each, but for simplicity, only the group 4 chromosomes are shown. The position of the B-locus is indicated by a black bar. This bar is omitted in panels 2 and 3 where the B-locus is presumed inactive or deleted. Immediately following formation of the tetraploids by polyploidization (panel 2, left-hand side), the B-loci are present as in the diploid progenitors. However, genome rearrangements following polyploidization lead to the loss of some of the B-loci (panel 2, right-hand side). The cross between *Ae. peregrina* and KU37 (panel 3) results in an F<sub>1</sub> that is heterozygous for the B-locus on chromosome 4S. The B-locus on chromosome 4U is inactive. Thus, although the *Aegilops* used in this study are tetraploid, only a single B-loci is responsible for the initiation of B-granules.

related to *Ae. longissima* (S<sup>1</sup>) and *Ae. umbellulata* (U) (Zhang *et al.*, 1992; Kimber and Yen, 1989). Although the original diploid progenitor accessions are unknown, it is safe to assume that they had normal B-granule content similar to all present-day diploid *Aegilops* species. Therefore, it is expected that the U and the S<sup>1</sup> progenitor genomes each possessed the major locus on chromosome 4 determining the presence of B-granules. It is proposed that *Ae. peregrina* probably lost the ability to make B-granules shortly after the tetraploidization event and that both of the homoeologous B-granule loci were inactivated, either because of gene loss, epigenetic silencing or mutation. The active alleles at these loci will be called B<sup>U</sup> and B<sup>S</sup> and the inactive alleles, b<sup>U</sup> and b<sup>S</sup>. In the mapping population derived from the cross between KU37 and *Ae. peregrina*, it is proposed that the presence of B-granules was determined primarily by the segregation of S-genome alleles (B<sup>S</sup> and b<sup>S</sup>) and that the U genome alleles in both KU37 and *Ae. peregrina* are inactive (b<sup>U</sup>). This model implies that, in the creation of the KU37 synthetic tetraploid, the B<sup>U</sup> homoeologue from *Ae. umbellulata* was inactivated whilst the B<sup>S</sup> homoeologue from *Ae. sharonensis* remained functional.

The proposed model assumes that there is a single locus per haploid genome controlling most of the variation in B-granule content and that there was no polymorphism at the B-granule locus on the U genome in our population. It is the simplest model that is consistent with our data. The major QTL on chromosome 4S and the segregation ratio of the F<sub>2</sub> phenotypes support the model and the fact that B-granule-less species of *Aegilops* appear to have evolved several times independently against complex genetic control. However, alternative models cannot be precluded at the moment. For example, it is possible that both B-loci in the synthetic are active and that there are two segregating loci in the F<sub>2</sub> population rather than one. The quantitative nature of the phenotypic segregation could also be taken to indicate the presence of multiple contributing loci. Alternatively, it may reflect the technical difficulty of accurately estimating the number of B-granules from measurements of average granule size. The size-distributions of A and B granules overlap in *Aegilops* more than in wheat or barley, making assignment of granule type based on granule size alone more precarious. In addition, small A-granules and B-granules are difficult to distinguish by shape, there is extensive variation in granule-size distribution from seed to seed within a genotype, and granule size is known to be affected by the environment. The most precise phenotyping method would be to assess the second granule initiation event which defines B-granules *per se*, but the determination of granule numbers through grain development (Fig. 2C, D) was impractical in the current study where large numbers of samples of half-seeds needed to be analysed. Despite the limitations outlined above, a major QTL determining B-granule content was identified which showed significant differences between allelic classes and explained a large proportion of the phenotypic variation. To test the model, a larger and more extended genetic map is currently being constructed.

The putative homoeologous genes that underlie the B-granule locus on the short arm of chromosome 4S and 4U in *Aegilops* have not yet been identified. Orthologues of the genes controlling B-granules in *Aegilops* are likely to be present in other Triticeae and there is evidence to suggest that these orthologues in wheat and barley may also be located on the group 4 chromosomes. In wheat, a QTL affecting A:B granule ratio was found on chromosome 4B (Batey *et al.*, 2001) and, in a separate study, QTLs associated with granule size were found on 4DS (Igrejas *et al.*, 2002). In barley, a QTL affecting the shape of the B-granules was found on chromosome 4H (Borém *et al.*, 1999). However, in all of these studies, QTLs influencing various aspects of granule size and/or shape were also associated with other chromosomes.

Our analysis suggests that the role of the B-granule gene in the Triticeae is to stimulate granule initiation since the recessive (mutant) allele conditions the lack of B-granules. Genetic analysis indicated that the active allele had an additive effect, meaning that the number of B-granule initiations increased with increasing gene dose. It remains possible that the B-granule locus also influences the number of A-granules that initiate since there were fewer A-granules in *Ae. peregrina* than in KU37. However, near-isogenic lines with and without B-granules will be needed to test this. It will be interesting to discover whether the initiation of A- and B-granules both require genes with similar functions or whether each process is different and also whether orthologues of the B-granule gene are found in grass species that do not possess A- and B-type granules, such as rice and *Brachypodium*.

Genes that have been shown to affect starch granule initiation and/or granule size in other plant species include isoamylase (Burton *et al.*, 2002; Bustos *et al.*, 2004), starch synthase IV (Roldán *et al.*, 2007) and starch phosphorylase (Satoh *et al.*, 2008). Of these, only starch synthase IV has been shown to stimulate granule initiation (isoamylase and phosphorylase inhibit granule initiation since loss of these activities leads to an increase in the number of small granules). Attempts to map the SSIV gene in *Aegilops* have so far failed due to lack of polymorphism in the available sequence, but it is not predicted from synteny to be located close to the B-granule locus. There is only one SSIV gene in *Brachypodium* (Bradi2g18810) and the wheat SSIV orthologue is located in a syntenous position on the long arm of chromosome 1 (Leterrier *et al.*, 2008). Thus, the B-granule locus in the Triticeae may encode a previously uncharacterized starch biosynthetic gene.

The genetic analysis suggests that fine mapping and eventual identification of the gene(s) responsible for B-granule initiation should be possible using this population of *Aegilops* and the phenotyping techniques described. Recombinant inbred lines from the *Aegilops* population are currently being produced to test this. Identification of the B-granule gene in the future will provide further understanding of granule-initiation in plants in general, as well as enabling manipulation of the A:B granule ratio in

domesticated Triticeae to create variant starches with altered functionality.

## Supplementary data

Supplementary data can be found at *JXB* online.

**Fig S1.** Variations in *Ae. peregrina* and KU37 plant morphology.

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