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Starch granule morphology in oat endosperm

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ABSTRACT

Mature and developing oat (*Avena sativa*) grains were sectioned and image analysis methods used to estimate the starch granule-size distribution and morphology in endosperm cells. This showed that oat endosperm cells contain two types of starch granule: compound starch granules such as those seen in rice endosperm and in most other grasses; and simple granules similar to the B-type starch granules seen in the endosperm of Triticeae species such as wheat (*Triticum aestivum*). The simple granules in oats are similar in size and relative abundance to B-type granules in Triticeae suggesting that they may share a common evolutionary origin. However, there is a fundamental difference between oats and Triticeae in the timing of granule initiation during grain development. In Triticeae, the B-type granules initiate several days after the A-type granules whereas in oats, both the simple and compound granule types initiate at the same time, in early grain development.

1. Introduction

The starch granules in the endosperm of most grasses are compound (Matsushima, 2015) i.e. they are composed of multiple, separately-initiated granules that have grown together within the same plastid and partially fused. Oat (*Avena sativa*) endosperm contains compound starch granules, but unusually for grasses, oat endosperm cells also contain smaller, simple starch granules (Fulcher, 1986; Buléon et al., 1998). The simple granules pack between the larger, compound granules.

The Aveneae and the Triticeae tribes are closely related taxonomically and unlike other grasses, both have two types of starch granule that differ in size and morphology in their endosperm cells. The small granules in oats are simple (i.e. generated from a single initiation) and they appear from microscopy to be quite similar in shape (roughly spherical) to the B-type starch granules seen in the endosperm of wheat (*Triticum aestivum*) and other members of the Triticeae tribe (Fulcher, 1986; Verhoeven et al., 2004). The large granules in oat endosperm, however, are very different in morphology from the large A-type granules in Triticeae. In oats, the large granules are ovoid in shape and compound (comprising many separately-initiated and partially-fused sub-granules) whereas in Triticeae such as wheat (Evers, 1973), the large granules are disc-shaped and simple (comprising a single granule). The compound granules in oats have sometimes been referred to as A-type granules and the simple granules as B-type (Buléon et al., 1998). However, due to the different morphologies of the large granules in wheat and oats, here, we will refer to the two sorts of granules in Triticeae as A- and B-type but to those in oats as 'compound' and 'simple'.

As well as varying in size and shape, the A- and B-type granules in Triticeae also vary in the timing of their initiation during grain development (Briarty et al., 1979) and in their position of initiation within the plastid (Wei et al., 2010). A-type granules form early in grain development and B-type granules form approximately 8 days later (Wei et al., 2010). The A-type granules form in the main body of the plastid whereas the B-type granules form in tubular protrusions called stromules (Buttrose, 1963; Parker, 1985; Langeveld et al., 2000; Bechtel and Wilson, 2003). We have as yet no information for oat endosperm about the timing of granule initiation or position within plastids of the compound and simple granules.

When extracted, oat starch has a unimodal granule-size distribution, unlike extracted wheat and barley (*Hordeum vulgare*)

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starches which show bimodal distributions. This is due to the fragility of the oat compound granules: when extracted from mature endosperm, the compound granules fall apart (largely but not completely) into their component granule parts. The component parts of the compound granules are similar in size (3–6 μm in diameter) (Tester and Karkalas, 1996; Wang and White, 1994) to the simple granules in oats (4–10 μm in diameter) (Fulcher, 1986). Thus techniques which rely on extracted starch (such as laser-diffraction particle-size analysis) cannot be used to accurately determine the relative size and number of the compound and simple granules in oat endosperm. For this reason, we have developed a technique based on microscopy and image analysis of starch granules *in situ* in sections of oat grain to measure their dimensions and relative numbers. We have used this to compare the granule-size distribution and pattern of granule initiation during grain development in oats with that in Triticeae species.

2. Experimental

2.1. Plant material

Two commercially-grown samples of mature oats were obtained for analysis. Sample A was the cultivar 'Canyon' and had normal processing properties. Sample B was from a commercial batch of oats sourced from Finland (2012 harvest) which, following processing into flakes, exhibited poor cooking properties. Analysis by rapid visco analysis (RVA) and differential scanning calorimetry (DSC) (data not shown), indicated differences in starch pasting properties and starch gelatinization between this batch and typical UK-sourced oats. Whether there were additional alterations to other components of the Sample B grains, such as the bran, cell walls (e.g.beta-glucans) or protein, is not yet known. Immature grain samples were obtained from oat cultivar 'Canyon' grown in trial plots at NIAB, Noon Folly Farm, Bar Hill, Cambridgeshire, UK in 2015.

2.2. Sampling developing grains

Plants growing within the plot rather than those at the edges were chosen for sampling immature, developing grains. For each plant, one panicle (usually the first panicle to emerge) was tagged at Growth Stage 59 (GS59; as defined in cereal growth stages: a guide for crop treatments, http://cereals.ahdb.org.uk). On this day (Day 0), the panicle had fully emerged i.e. the lower-most spikelet was above the ligule of the flag leaf. Thereafter, samples were harvested at various times between 3 and 42 days after Day 0. The harvested panicles were taken to the lab as cut stems in a vase of water. In the lab, the distance between the lower-most spikelet and the ligule was measured and 4 or 6 (depending on size) primary grains were removed from the top-most 4–6 spikelets. The number of grains per sample and their total fresh weight was recorded. Replicate samples were photographed and then discarded.

2.3. Microscopy

Microscopy was performed by Rothamsted Bioimaging (www.rothamsted.ac.uk/bioimaging). Mature oat grains were soaked in distilled water overnight, cut in half transversely and fixed for 16–24 h in fixative (4% (v/v) paraformaldehyde, 0.25% glutaraldehyde, 50 mM phosphate buffer, pH 7.2). Samples were washed in 0.1 M phosphate buffer, pH 7.2, dehydrated through an ethanol series, and then infiltrated with LR White resin over 3–4 days. Samples were polymerised in fresh resin in capsules for 16–20 h at 60 °C. The resin blocks were sectioned using a Leica UC6 ultramicrotome and 1 μm sections mounted on polysine-coated slides (Thermo Fisher Scientific). Sections were stained with either 0.1% Toluidine Blue (Sigma-Aldrich) to reveal the general cellular arrangement of the endosperm or with diluted Lugol's iodine solution (Sigma-Aldrich) to reveal starch granules. Sections were viewed using a Leica DM2500 microscope and DFC420C camera.

Developing oat grains (whole or for large grains, cut in half transversely) were placed in 2–4 ml of fixative in a 7-ml TAAB glass vial, vacuum infiltrated 2–3 times and then incubated at 4 °C for up to 1 month (whilst harvesting of the entire developmental series was completed). Samples were washed, dehydrated, infiltrated with LR White resin (Sigma-Aldrich) and 1 μm sections prepared for microscopy as above for mature grain sections.

2.4. Image analysis

Iodine-stained sections were viewed whilst the staining was fresh and the starch granules appeared black against an unstained background. Sections that were well-fixed and devoid of creases were chosen. Micrographs of endosperm cells in the centre of the lobes were taken using a 20× or 40× objective (Fig. 1). High-resolution images were stored as jpeg files.

ImageJ software (http://imagej.nih.gov/ij/) was used to analyze the micrographs as follows: individual cells within the section were chosen (Fig. 1A) and their area determined. For each cell, the image outside the cell was deleted and the image was made binary (starch black against white background). The total starch area was measured. To determine the area of the compound and simple granules separately, the original micrographs were printed and the outlines of either the simple or the compound granules in the chosen cell were traced onto tracing paper. The granule outlines were coloured in and the 2 tracings (one of the simple and the other of the compound starch granules in the cell) were scanned to digitize them (Fig. 1B and C). The areas (individual and total) of the compound and simple granules were determined using ImageJ. Statistical analyses (Student's *t*-test) were performed using Microsoft Excel.

3. Results

3.1. Mature grains

To study the starch granule-size distribution in mature oat endosperm, we used image analysis of starch granules in micrographs of grain sections (Fig. 1). Single endosperm cells were analyzed so that the mean starch content per cell and the mean cell size could be determined as well as the ratio of simple to compound granules and the relative and absolute sizes of these granules. Measurements were made for two genotypes: Sample A which had normal processing properties and Sample B which processed poorly. The measurements and statistical comparisons are shown in Table S1. The mean values per sample are also shown graphically in Fig. 2.

To ensure that representative samples of cells and starch were chosen for each sample, six replicate grains were sectioned per sample. Transverse sections were taken from approximately the midpoint along the length of the grain. From the multiple sections of each grain, five endosperm cells were chosen for analysis (i.e. 30 cells per sample). These cells were from the centre of the endosperm lobes. There was no statistically-significant difference between the two samples in average cell area (Fig. 2A, Table S1). To check that the starch areas per cell determined from the drawings (compound and simple granules) and micrographs (total starch) were comparable, the total starch areas per cell were calculated using both methods and compared (Fig. 2B, Table S1). There was no statistically-significant difference between the values obtained using the two methods.