Grain development in *Brachypodium* and other grasses: possible interactions between cell expansion, starch deposition, and cell-wall synthesis

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**Abstract**

To explain the low levels of starch, high levels of (1,3;1,4)-β-glucan, and thick cell walls in grains of *Brachypodium distachyon* L. relative to those in other Pooidae, aspects of grain development were compared between *B. distachyon* and barley (*Hordeum vulgare* L.). Cell proliferation, cell expansion, and endoreduplication were reduced in *B. distachyon* relative to barley and, consistent with these changes, transcriptional downregulation of the cell-cycle genes CDKB1 and cyclin A3 was observed. Similarly, reduced transcription of starch synthase I and starch-branching enzyme I was observed as well as reduced activity of starch synthase and ADP-glucose pyrophosphorylase, which are consistent with the lowered starch content in *B. distachyon* grains. No change was detected in transcription of the major gene involved in (1,3;1,4)-β-glucan synthesis, cellulose synthase-like F6. These results suggest that, while low starch content results from a reduced capacity for starch synthesis, the unusually thick cell walls in *B. distachyon* endosperm probably result from continuing (1,3;1,4)-β-glucan deposition in endosperm cells that fail to expand. This raises the possibility that endosperm expansion is linked to starch deposition.

**Key words:** *Brachypodium*, (1,3;1,4)-β-glucan, cell cycle, cell wall, endosperm, starch.

**Introduction**

*Brachypodium distachyon* is a grass species native to southern Europe, northern Africa, and southwestern Asia. It is a self-fertile annual with a rapid life cycle and a relatively small stature. The genus *Brachypodium* belongs to the subfamily Pooidae of the grasses (Poaceae), which also includes the grain crops barley (*Hordeum vulgare* L.), wheat (*Triticum aestivum* L.), rye (*Secale cereal* L.), and oats (*Avena sativa* L.). *B. distachyon* was the first Pooidae species to have its genome sequenced and has become a model system for temperate cereals, forage grasses, and biofuel grass crops (Draper et al., 2001; Brkljacic et al., 2011; Vain, 2011).
In grains, starch is usually the major storage carbohydrate for immediate post-germinative growth, while β-glucan is a component of the cell walls of grasses (Carpita, 1996; Yokoyama and Nishitani, 2004; Fincher, 2009). However, in *B. distachyon* grains, β-glucan is the major grain carbohydrate and, as well as having a structural role, may supplement starch as a storage carbohydrate (Guillon et al., 2011; Opanowicz et al., 2011).

The unusual grain composition of *B. distachyon* suggests a shift in carbohydrate metabolism from starch to β-glucan compared with that of closely related grasses. The primary cause of this metabolic shift is not known. Both starch and β-glucan are synthesized from nucleotide sugars (ADP-glucose and UDP-glucose, respectively) that are produced in developing endosperm cells from the products of sucrose breakdown (Kleczkowski, 1996). The cytosolic UDP-glucose pool is common to both pathways, and there is some evidence for competition between the starch and β-glucan pathways for this substrate. For example, in barley, mutations that directly affect starch accumulation in the endosperm lead indirectly to increases in β-glucan content (Trafford and Fincher, 2013). It is therefore possible that restrictions in starch biosynthesis in *B. distachyon* compared with other grass species lead, secondarily, to an increase in β-glucan synthesis. Alternatively, an increase in the capacity for β-glucan synthesis in *B. distachyon* relative to other grasses could lead to a decrease in starch synthesis due to competition for the common nucleotide sugar substrate.

Grain morphology, anatomy, and development in *B. distachyon* have been compared with those of other species (Guillon et al., 2011; Opanowicz et al., 2011; Hands and Drea, 2012; Hands et al., 2012). While the grains of *B. distachyon* are broadly similar to those of wheat and barley, there are a number of distinct features. In *B. distachyon*, the endosperm cells are relatively undifferentiated and the cell walls are very much thicker than those in other species. However, in composition, the cell walls in *B. distachyon* are similar to those in barley with β-glucan being the main component of the endosperm cell walls in both species (barley 75%, w/w; *B. distachyon* 80%, w/w) (Guillon et al., 2011). A survey of grain morphology showed that, in addition to *B. distachyon*, two species of *Bromus* also have thick endosperm cell walls, but this feature was not seen in any of the other six grasses studied (Hands et al., 2012). This suggests that thick cell walls and the associated high β-glucan content may be restricted to certain genera within the Pooidae.

Here, we have further examined the extent of variation in grain composition among the genera *Brachypodium* and *Bromus* and among other Pooidae. We have explored the factors responsible for variation in the starch:β-glucan ratio in grains using *B. distachyon* and barley as examples of extreme types. We considered both metabolic and cell-developmental explanations, examining the content and expression of genes involved in carbohydrate metabolism and the activities of critical enzymes as well as the extent of cell-wall thickening relative to endosperm cell expansion.

**Materials and methods**

**Plants, grain samples, and growth conditions**

*B. distachyon* Bd21 was grown in a controlled environment room under full-spectrum fluorescent lights at 22 °C with a 20 h light and 4 h dark cycle and 60% relative humidity. Barley was grown in a controlled environment room at a constant temperature of 15 °C with a 16 h light and 8 h dark cycle and 70% humidity or in a glasshouse at a minimum temperature of 16 °C and a minimum of 12 h light. The other Pooidae accessions used are described in Supplementary Table S1 at *JXB* online.

**Glucan assays**

For mature grains, starch and β-glucan contents were measured using Total Starch and Mixed-Linkage Beta-Glucan kits (Megazyme International) according the manufacturer’s instructions, except that the extraction procedures were combined and the assays scaled down to accommodate small sample sizes, as follows. Grains (1–20 grains with total mass of 4.5–60 mg) were incubated with 0.5 ml of 0.3% sodium metabisulphite and 1% lactic acid (pH 3.8) at 52 °C overnight. The steep liquid was discarded. The grains were blotted dry, cut into small pieces with scissors, and ground in a mortar with a pestle in 0.5 ml of extraction buffer (20 mM sodium phosphate, pH 6.5). The extract was diluted with extraction buffer to give ~0.1 ml mg⁻¹ of seed dry weight. An aliquot of 0.8 ml was transferred to a screw-capped tube and autoclaved at 121 °C for 15 min to solubilize the starch and β-glucan. Three 50 μl aliquots of each extract were digested as follows. Aliquot 1 was mixed with 50 μl of starch digest buffer (containing 100 mM sodium acetate (pH 5.2), 5 mM CaCl₂, 0.3 U α-amylase and 1 U of amyloglucosidase). Aliquot 2 was mixed with 50 μl of 100 mM sodium acetate (pH 5.2). Aliquot 3 was mixed with 0.1 U of lichenase, incubated at 37 °C for 1 h, and then mixed with 50 μl β-glucan digest buffer (containing 50 mM sodium acetate (pH 4.0) and 0.1 U of β-glucosidase). All three aliquots were incubated at 37 °C overnight and then assayed for glucose using the glucose oxidase peroxidase reagents supplied in the Megazyme kits. The glucose assays, in a final volume of 0.8 ml, contained 0.75 ml GOPOD reagent and 50 μl water plus extract and were incubated at 50 °C for 20 min, allowed to cool, and then measured for absorbance at 510 nm. The assay was calibrated using a standard curve of 0–30 μg glucose. At least three individual samples of each grain type were separately extracted and digested, and assayed for glucose in duplicate.

The reliability of the small-scale assay protocol was tested by measuring the starch and β-glucan standards supplied with the Megazyme kits and by testing single-grain samples of *B. distachyon*, *Bromus diandrus*, and *Bromus rigidus* by both the small-scale and Megazyme-recommended protocols. The values obtained with the small-scale assay were 92–118% of the expected values.

For developing *B. distachyon* grains, the extraction and assay were as above except that 7–19 grains were harvested per sample, immediately frozen in liquid nitrogen and then ground in 0.5–1 ml of ice-cold 20 mM sodium phosphate (pH 6.5) prior to autoclaving.

**Enzyme assays**

For ADP-glucose pyrophosphorylase (AGPase) assays, approximately 100 mg of developing endosperm was homogenized in 1 ml of extraction buffer [50 mM Bis-Tris propane (pH 7.2), 5 mM KCl, 2 mM MgCl₂, 2 mM EDTA, 5% (v/v) ethanediol, 1 mM dithiothreitol (DTT), 10 μl ml⁻¹ of protease inhibitor cocktail (Sigma), 1% (w/v) BSA]. After centrifugation for 3 min at 10 000g, the supernatant was assayed for AGPase activity in the direction of ADP-glucose synthesis at 25 °C. Assays were duplicated, and each contained, in a volume of 1 ml, 5–250 μl of extract, 100 mM HEPES (pH 7.5), 5 mM MgCl₂, 0.8 mM NADP, 1% (w/v) BSA, 5 mM phosphoglyceric acid, 1.5 mM pyrophosphate, 1 mM ADP-glucose, 3 U of glucose 6-phosphate dehydrogenase, and 2 U of phosphoglucomutase. The amount of...
extract was optimized and the rate of reaction was linear with respect to time for at least 15 min. At least three separate extracts of each species were assayed.

For starch synthase (SS) assays, 40–65 mg of developing grains was homogenized in 1 ml of extraction buffer [100 mM HEPES (pH 7.5), 5 mM DTT, 1% (v/v) BSA]. After centrifugation for 5 min at 14,000g, the supernatant was assayed for SS according to the method of Jenner et al. (1994) (Dowex method). Assays were duplicated and each contained, in a volume of 0.1 ml, 100 mM Bicine (pH 8.5), 5 mM EDTA, 25 mM potassium acetate, 10 mM DTT, 1 mM ADP-glucose, 0.23 KBq ADP[U-14C]glucose, 5 mg ml–1 of potato amylopectin, and 10 μl of extract. The amount of extract was optimized and the rate of reaction was linear with respect to time for at least 10 min. Assays were incubated at 25 °C for 0 min (controls), 10 min (barley) or 20 min (B. distachyon) before being terminated by heating to 100 °C for 2 min. At least six separate extracts of each species were assayed.

**Light microscopy**

To localize the starch distribution within mature grains (see Fig. 2), grains that had been stored in ethanol were soaked in distilled water overnight. Thin transverse sections were cut with a razor blade at approximately 1 mm intervals along the grain and stained with Lugol’s solution, and examined and photographed with an Olympus BX60 with ProgRes® Capture Pro 2.1 software (Jenoptik). Grains were also cut longitudinally, stained with Lugol’s solution, and imaged using a dissecting microscope.

Immature grains were harvested at specific stages of development. To facilitate fixation, grains were cut in half or their ends removed. The dissected grains were fixed for 16 h in 4% (v/v) paraformaldehyde, 0.25% (v/v) glutaraldehyde, and 4% sucrose in PBS. Samples were washed overnight in PBS, dehydrated in a graded ethanol series, and slowly infiltrated with LR White resin over several days. Individual grains were placed in gelatin capsules, which were filled with fresh resin and polymerized for 48 h at 55 °C. Resin blocks were sectioned (1 μm) on a Leica EM UC6 ultramicrotome. The sections were collected, dried onto glass microscope slides, and stained with 0.01% (w/v) toluidine blue or with Lugol’s solution. Images were analysed using ImageJ software (http://rsbweb.nih.gov/ij/index.html) as described in the figure legend.

**Analysis of endoreduplication**

Grain samples (two grains for barley, five grains for B. distachyon) were chopped in 1 ml of lysis buffer (LB01 buffer minus spermine tetrahydrochloride; Dolezel et al., 1989) in a plastic Petri dish using a single-edged razor blade. After gravity-based filtration through a 40 μm Filcon (BD Biosciences), propidium iodide (Sigma) was added to a final concentration of 50 μg ml–1. The integrity of the stained nuclei was verified microscopically (excitation BP 545/25, emission BP 605/70). Within 1 h, samples were analysed on a BD Aria Flow Cytometer using FACS Diva Software version 6.1.3 (BD Biosciences). For each sample, a minimum of 1000 stained nuclei were sorted. Data were analysed using FCS Express for Flow Cytometry version 4 (De Novo Software, Los Angeles, CA, USA).

**RNA isolation and cDNA synthesis**

Spikes of barley and individual grains of B. distachyon were tagged at anthesis and the developing caryopses were collected at regular intervals after cellularization was complete and until maximum fresh weight was attained. For barley, the pericarp and the embryo were removed, leaving the cellularized endosperm. For B. distachyon, whole grains were collected. All tissues were immediately frozen in liquid nitrogen and stored at −80 °C prior to RNA extraction. Multiple endosperms from at least three different spikes were collected and combined before RNA extraction.

Total RNA was extracted from all tissue homogenates using a phenol/guanidine reagent, treated with a DNA-free kit (Ambion), and used as template for cDNA synthesis as described by Burton et al. (2004) except that Superscript III reverse transcriptase (Invitrogen) was used in preference to Superscript II and the reaction was incubated at 50 °C.

**Transcript analysis**

Quantitative real-time PCR (qPCR) primers were designed to target the same area of every gene (Supplementary Table S2 at JXB online). Generally, one primer was located in the 3’ exon and the other in the 3’ untranslated region. This strategy combines the specificity of the 3’ untranslated region and the high signal of the 3’ of the transcript. Real-time qPCR templates were purified, quantified, and sequenced as described by Burton et al. (2008). qPCR was carried out essentially as outlined by Burton et al. (2004) with the following modifications. To provide a template for the standard curve, between four and six 20 μl PCR mixtures were combined for purification by HPLC using a HELIX DNA DVB 50-3 3.0 mm monolithic polymer reversed-phase column (Varian). Chromatography was performed using buffer A [100 mM triethylammonium acetate (Applied Biosystems) and 0.1 mM EDTA] and buffer B (100 mM triethylammonium acetate, 0.1 mM EDTA, and 75% acetonitrile). The gradient was as follows: time 0 min, 10% buffer B; time 6 min, 21.5% buffer B; time 7 min, 21.5% buffer B; time 8 min, 10% buffer B; time 12 min, 10% buffer B. The flow rate was 0.45 ml min–1 and the temperature was 50 °C.

Each qPCR experiment consisted of three replicates of seven standard concentrations as well as water, together with three replicates of each cDNA sample. Each individual qPCR contained 2 μl of either a cDNA solution, a standard, or water together with a master mix containing 1.2 μl of forward and reverse primers, 0.3 μl of water, and 5 μl of Kapa SYBR FAST qPCR master mix (Kapa Biosystems). These qPCRs were assembled by a liquid-handling CAS-1200 robot (Qiagen) and run on a Mastercycler ep realplex QPCR instrument (Eppendorf). The PCR cycle was as follows: 3 min at 95 °C, followed by 40 cycles of 10 s at 95 °C, 10 s at 55 °C, and 30 s at 72 °C, and 15 s at 1 °C below the optimal acquisition temperature (T_m) described in Supplementary Table S2. Fluorescence data were obtained at this temperature where it was expected that off-target, non-specific, double-stranded material had melted. Melt curves were obtained as follows: 15 s at 95 °C and 15 s at 60 °C, and then 95 °C for at least 20 min.

Data were analysed using realplex software (Eppendorf). Transcript levels in all cDNAs were calculated with respect to the qPCR standards and the SEM was calculated from the three replicate qPCRs. To assess relative gene expression in two different species, we employed the geNorm method of Vandesomple et al. (2002). Previously, we have used this method to calculate ‘normalized transcript levels’ in sets of cDNAs from one species (Burton et al., 2004). In this case, we extended the analysis to include transcripts from two species. Data were normalized against the geometric means of the three out of four control genes that varied the least with respect to each other across the two species. Concentrations of transcripts are given in arbitrary units that represent the numbers of copies μl–1 of cDNA relative to the concentrations of the controls.

**Results**

Brachypodieae and Bromeeae have distinct grain compositions that differ from one another and from those of other Pooidae

To discover the extent to which the high β-glucan/low starch grain composition of B. distachyon was shared with related species, we surveyed the composition of three different accessions of B. distachyon, several Brachypodium and Bromus species, and a selection of other Pooidae that together
represent six of the 12 tribes of the Pooidae (Fig. 1A, B and Supplementary Table S1). The unusual grain composition of *B. distachyon* Bd21 was also found in all other *Brachypodium* species analysed. All of the *Bromus* species also had a distinct ratio of starch:β-glucan but this was different from the ratio found in the *Brachypodium* species. Apart from members of the Brachypodieae and Bromeae tribes, all other Pooidae species had relatively high starch and low β-glucan contents. The values of the starch:β-glucan ratios observed fitted to a curve (power regression, correlation coefficient $R^2=0.69$; data not shown), suggesting that there is an inverse correlation between starch and β-glucan content across a wide range of Pooidae species.

**Starch is unevenly distributed in the endosperm of mature B. distachyon grains**

The spatial distribution of starch in whole *B. distachyon* grains was examined using thick transverse and longitudinal sections of mature grains stained with Lugol’s solution (Fig. 2A). This showed that starch was more abundant at the proximal end of the endosperm, close to the embryo, than at the distal end. At the distal end, starch was less abundant in the lobes of the endosperm and was primarily confined to the central zone. Corresponding longitudinal sections of mature wheat and barley grains showed no detectable gradient in starch content from proximal to distal ends (data not shown). Biochemical measurements of starch and β-glucan content in the middle and distal portions of the grain confirmed the unequal distribution of starch in the endosperm and showed that β-glucan content was highest in the distal endosperm where starch content was lowest (Fig. 2B).

**Starch content is low throughout endosperm development**

To characterize the development of *B. distachyon* grains under our growth conditions, replicate samples were taken for microscopy (Figs 3A and 4) and biochemical analysis (Fig. 3B). Microscopic examination showed that cellularization of the endosperm occurred between 7 and 8 d after pollination (DAP) and maximum fresh weight was achieved at approximately 19 DAP. Prior to endosperm cellularization, starch was present mainly in the pericarp (data not shown). After cellularization, the starch content of the pericarp declined while that in the endosperm increased. Immediately after cellularization, the endosperm cells had thin walls and were devoid of starch (Fig. 3A, i). After 8 DAP, the endosperm cell walls became increasingly thickened and cells in the central endosperm became filled with starch granules (Fig. 3A, ii, iii).

Characterization of *B. distachyon* grain composition showed that, under long-day growth conditions, as in short days (Guillon *et al.*, 2012), both starch and β-glucan increased steadily in the amount in the endosperm throughout the post-cellularization phase of development (Fig. 3B). The starch content of whole grains was low at all stages of development. In contrast, β-glucan content increased steadily throughout grain development, as observed previously for barley (Wilson *et al.*, 2012). The rate of starch synthesis in the endosperm of *B. distachyon* was estimated assuming that (i) at 18 DAP starch was entirely in the endosperm, and (ii) at 8 DAP starch content in the endosperm was zero. From this, it could be calculated that the rate of starch synthesis per grain was approximately 7-fold lower than the rate of β-glucan synthesis.

*B. distachyon* possesses all of the genes required for starch synthesis

The starch-related genes in *B. distachyon* were compared with those in rice (*Oryza sativa L.*) (Table 1). This showed that orthologues of all of the rice starch genes except two were present in the *B. distachyon* genome. The two genes absent from *B. distachyon* encode, respectively, a starch synthase IV (SSIV) and a subunit of AGPase. There are two

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**Fig. 1.** Starch and β-glucan contents of mature Pooidae grains. (A) Starch and β-glucan contents of mature whole grains from a range of Pooidae species (see Supplementary Table S1) representing six of the 12 Pooidae tribes. Values are means for at least three separate extracts each assayed in duplicate. (B) Taxonomic relationships between the Pooidae tribes in (A), based on data from Catalán *et al.* (1997). (This figure is available in colour at JXB online.)
SSIV genes in rice, but only one in B. distachyon. However, all other grasses with sequenced genomes also have only one SSIV orthologue (http://www.phytozome.net; Goodstein et al., 2012). Thus, rice rather than B. distachyon is unusual in this respect.

Regarding AGPase, there are four genes encoding AGPase large subunits in rice but orthologues of only three of these were present in B. distachyon. The gene not represented in B. distachyon is called OsAPL2 or OsAGPL2 in rice and Shrunken2 in maize, and encodes the major cytosolic large subunit of AGPase in the endosperm (Lee et al., 2007). Mutants of rice and maize affected in this gene have severely restricted starch content (Giroux and Hannah, 1994; Lee et al., 2007). However, like B. distachyon, neither wheat nor barley possesses genes of this type (Rösti, 2006). As the starch content of barley and
wheat is similar to that of other cereals, this shows that an *OsAPL2/Shrunken2* orthologue is not required for normal starch synthesis in at least some, perhaps all, of the Pooidae. Its absence from *B. distachyon* does not, therefore, necessarily explain the low rate of starch synthesis in this species.

Table 1. Comparison of rice and *B. distachyon* genes involved in starch synthesis

<table>
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<tr>
<th>Enzyme</th>
<th>EC no.</th>
<th>CAZy family</th>
<th>Name</th>
<th>Alternative name</th>
<th>Rice gene (MSU/TIGR)</th>
<th><em>B. distachyon</em> orthologue (modelcrop.org)</th>
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<sup>a</sup>Nozue et al. (1996); <sup>b</sup>Akihiro et al. (2005); <sup>c</sup>Ohdan et al. (2005); <sup>d</sup>Toyota et al. (2006); <sup>e</sup>Lee et al. (2007); <sup>f</sup>Hirose et al. (2006); <sup>g</sup>Dian et al. (2005); <sup>h</sup>Okagaki (1992); <sup>i</sup>Nishi et al. (2001); <sup>j</sup>Comparat-Moss and Denyer (2009); <sup>k</sup>Rösti et al. (2007).
The activity of starch biosynthetic enzymes is reduced in B. distachyon endosperm

The activity of starch biosynthetic enzymes is reduced in B. distachyon endosperm. The maximum catalytic activities of two enzymes required for starch biosynthesis, AGPase and SS, were measured in developing grains of B. distachyon and barley. The activity (mean ±SEM) of AGPase in B. distachyon (0.2 ± 0.1 μmol min⁻¹ g⁻¹ of fresh weight) was at least an order of magnitude lower than that in barley (3.7 ± 1.7 μmol min⁻¹ g⁻¹ of fresh weight), while SS activity in B. distachyon (37.2 ± 1.4 nmol min⁻¹ g⁻¹ of fresh weight) was less than half of that in barley (97.3 ± 8.8 nmol min⁻¹ g⁻¹ of fresh weight). The activities of barley AGPase and SS were within the range of values found previously for this species and for grasses in general (AGPase: 1-8 μmol min⁻¹ g⁻¹ of fresh weight; SS: 60-200 nmol min⁻¹ g⁻¹ of fresh weight) (Ozbun et al., 1973; Jenner et al., 1994; Thorbjørnsen et al., 1996; Beckles et al., 2001; Tetlow et al., 2003; Patron et al., 2004).

Cell division and expansion are limited in developing B. distachyon endosperm

To compare grain development in B. distachyon and barley, samples harvested at various DAP were fixed, sectioned transversely, and observed microscopically (Fig. 4). Image analysis was used to measure the areas of the endosperm, starch granules, non-starch cytoplasm, and cell walls, and also to estimate the number of cells across the endosperm lobes (Fig. 5). This showed that the mid-grain cross-sectional area of B. distachyon endosperm increased 2.5-fold over the grain-filling period (from 8 to 19 DAP; Fig. 5A). In comparison, the increase in cross-sectional area in barley over the same period was >15-fold (Fig. 5A). The increase in barley endosperm size is similar to that seen previously; for example, a value of 11-fold can be calculated from the figures in Gubatz and Shewry (2011).

In B. distachyon, the number of cells across the endosperm lobe varied very little between 8 and 19 DAP (Fig. 5B). In contrast, the number of cells across the lobes of barley endosperm increased nearly 2-fold over the same period of development (5–24 DAP). Thus, the increase in endosperm area in B. distachyon is mainly attributable to an increase in average cell area rather than to an increase in cell number. However, the endosperm cells in B. distachyon enlarged far less than those of barley during the course of development (2-fold compared with 6-fold in barley) (Fig. 5C).

In B. distachyon, the small increase in cell area was due mainly to thickening of the cell wall but also to the accumulation of a small amount of starch (Fig. 5C). In barley, the increase in cell area during development was accounted for by the increasing area occupied by starch. The endosperm cell walls in barley remained thin throughout development and their contribution to total cell area was minimal. To estimate the area within the cell wall that was not occupied by starch, the total area of starch granules per cell was subtracted from the area bounded by the cell wall. This non-starch cytoplasmic area changed very little over development in either B. distachyon or barley (Fig. 5C).

Evidence that endoreduplication in B. distachyon endosperm is limited

Given the low rate of cell proliferation in B. distachyon compared with barley, we compared the DNA content (ploidy level) of the endosperm cells. Cell flow cytometry was used to compare the DNA content of nuclei from leaves and grains of both B. distachyon and barley (Fig. 6). The leaves give a simpler profile, which can be used to calibrate the more complex grain profiles. This study also indicated the extent of endoreduplication that had occurred in the nuclei. Endoreduplication is commonly seen in the later stages of endosperm development, accompanying storage product accumulation (Sabelli and Larkins, 2009). It is also observed in other metabolically active, terminally differentiated plant tissues (Larkins et al., 2001). Nuclei were prepared from developing grains of both species at three developmental stages: pre-cellularization, post-cellularization, and mature.

Fig. 4. Light micrographs of developing B. distachyon and barley grains. Grains were fixed, sectioned transversely, and stained with toluidine blue. (This figure is available in colour at JXB online.)
In both species and organs, the predominant ploidy level observed was 2C (i.e. twice the DNA content of the haploid nucleus). In the grain, these 2C nuclei are from the diploid embryo/maternal tissues. Nuclei with higher ploidy levels were seen in all samples. In the grain, the 3C nuclei derived from the triploid endosperm. Nuclei with 4C and 6C ploidy levels were diploid or triploid nuclei, respectively, that had undergone DNA replication. Such doubling of DNA content is seen prior to cell division (during the S phase of mitosis) or in cells beginning the process of endoreduplication (Kowles and Phillips, 1988; Stals and Inzé, 2001; Inzé and De Veylder, 2006). The presence of 6C nuclei in *B. distachyon* endosperm suggested that cells are able to replicate their DNA, even though there was little evidence for cell division as the cell number in the endosperm remained constant (Fig. 5B).

Ploidy levels of 8C and 12C indicate endoreduplication of previously diploid or triploid cells, respectively. In the grains of barley, 12C nuclei were clearly observed in samples from grains at the post-cellularization and mature stages of development (Fig. 6) but not in grains at the pre-cellularization stage (data not shown). This indicated that, during grain development, at least some of the barley endosperm cells had undergone endoreduplication. However, relatively few, if any, 12C nuclei were observed in *B. distachyon* grains at any stage of development.

**Comparison of gene expression in B. distachyon and barley grains**

qPCR was used to compare the abundance of selected transcripts in *B. distachyon* grains with that of their orthologues in barley. Transcripts for genes associated with starch synthesis, cell-wall synthesis, sucrose catabolism, and cell cycling were examined. The data are given in Supplementary Tables S3 and S4 at *JXB* online.
To assess relative gene expression between the two species, the amount of each transcript of interest in a cDNA sample was expressed relative to that of certain control transcripts. These controls were chosen to represent genes likely to be expressed constitutively in both species. Similarly, Fowlkes et al. (2011) found certain transcripts in three Drosophila species to be ‘largely similar’ in abundance using a hybridization technique. While we cannot be sure that the control genes behave similarly in both species, we believe this method is the best way to reveal the gross differences in gene expression that exist between in B. distachyon and barley.

To give an overview of comparative gene expression, the maximum values of relative transcript abundance attained during development were compared (Fig. 7A). A very similar graph was obtained if the average transcript abundance values were plotted (data not shown). Most orthologous pairs were present at similar maximum levels in both species, with the abundance of 21 of the 36 transcript pairs examined differing between species by <10-fold. One gene with near-equivalent maximum (and average) transcript abundance in both species was cellulose synthase-like F6 (CslF6), the gene required for β-glucan synthesis in barley grains (Burton et al., 2006; Tonooka et al., 2009). No transcripts were more than 10-fold more abundant in B. distachyon than in barley. However, 15 transcripts were more than 10-fold more abundant in barley than in B. distachyon. The starch-related transcripts in particular were comparatively low in abundance in B. distachyon. Of the 10 starch transcripts examined, seven were >10-fold less abundant in B. distachyon than in barley. This compares with four of the eight cell-wall-related transcripts and three of the 12 cell-cycle-related transcripts.

Among the 36 transcripts examined, four were particularly strongly reduced in abundance (>300-fold) in B. distachyon compared with barley (Fig. 7A). These were SSI (2200-fold), starch-branching enzyme I (BEI; 580-fold), CDKB1 (300-fold), and cyclin A3 (350-fold). Comparison of transcript abundance through development showed that some of these transcripts pairs (SSI and BEI) had different patterns of expression between species as well as different concentrations, while others (CDKB1 and cyclin A3) had similar patterns of expression despite their different concentrations (Fig. 7B). The pattern of expression during development of CslF6 was
also found to be different between species even though the maximum (and average) amounts of CslF6 transcripts were similar. In B. distachyon, despite the near-constant accumulation of β-glucan during development, the amount of CslF6 transcript fell continuously.

Within each species, the pattern of expression of CDKB1 and cyclin A3 was very similar (Fig. 7B), suggesting that these genes are strongly co-expressed. Further analysis (data not shown) also revealed co-expression of CDKB2 with CslD4 in B. distachyon (correlation coefficient >0.95). The orthologue of B. distachyon CslD4 in maize is called CslD1 and has been shown to have a role in plant cell division (Hunter et al., 2012). Thus, a shared similarity of function in cell division may underlie the observed co-expression of these genes.

The amounts of sucrose synthase (SuSy) transcripts varied less than 10-fold between the two species. However, SuSy2 was the only transcript that was expressed more highly (>5-fold) in B. distachyon than in barley. Analysis of the patterns of expression of the SuSy transcripts through development (Fig. 7C) showed that in barley, SuSy1 was the most abundant of the three SuSy transcripts followed by SuSy3 and then SuSy2. In B. distachyon, this pattern was exactly reversed, with SuSy2 being the most abundant, followed by SuSy3 and then SuSy1.

Discussion

Unusual grain composition is a feature of members of the genus Brachypodium

The low starch:β-glucan ratio observed in B. distachyon is also found in other members of the Brachypodieae tribe but not in most other Pooidae. Members of the Bromeae tribe have starch:β-glucan ratios midway between those of the Brachypodieae and Triticeae. The Bromeae tribe is closely related to the Triticeae and belongs to a group called the core Pooids (Poaeae, Aveneae, Triticeae, and Bromeae), which are the most recently evolved Pooids (Catalán et al., 1997). The Brachypodieae diverged prior to evolution of the core Pooids (Fig 1B). Three of the core Pooids tribes do not share the low starch:β-glucan ratios observed for the Brachypodieae and Bromeae tribes. This suggests that the unusual grain compositions of the Brachypodieae and Bromeae evolved independently, rather than in a common ancestor of the Brachypodieae and core Pooids. To understand better the underlying reasons for variations in the starch:β-glucan ratio among different Pooidae species, B. distachyon and barley were studied in more detail, as examples of extreme types.

Evidence for a reduced capacity for starch synthesis in B. distachyon grains

Although B. distachyon possesses a full-complement of starch biosynthetic genes, we found evidence for reduced expression of some of these and/or reduced activity of the encoded enzymes in the developing endosperm. Reduced starch biosynthetic capacity in B. distachyon grains is therefore likely to account for the observed low starch content. In contrast, there was no increase in the capacity for β-glucan synthesis in B. distachyon endosperm, at least at the transcriptional level.

Starch synthesis may drive endosperm cell enlargement

Using measurements of cell cross-sectional area as a proxy for cell volume, we concluded that, in developing barley endosperm, the increase in cell volume is equivalent to the increase in the volume of starch per cell. Thus, it is possible that starch accumulation in barley drives cell expansion. Consistent with this, the relatively low level of starch accumulation in B. distachyon endosperm correlates with the comparative lack of cell expansion. There is a limited increase in cell size in B. distachyon during grain development, but this is due mainly to cell-wall thickening. Interestingly, the non-starch cytoplasmic volume remained
nearly constant throughout grain development in both barley and *Brachypodium*.

Increasing cell size during endosperm development has been described previously for both wheat and barley, and this, together with increasing cell number, accounts for endosperm expansion. In wheat, for example, endosperm cell number increases 5-fold, and both endosperm volume and cell volume increase 4-fold between the completion of cellularization and the end of grain filling (Briarty *et al.*, 1979). In *Brachypodium*, as well as limited cell expansion, cell number also increases relatively little during endosperm development. Consequently, in *Brachypodium* there is a relatively modest increase in endosperm size after cellularization.

**Thickened cell walls are a consequence of limited cell enlargement**

Our observations suggested that the unusually thick cell walls in *Brachypodium* endosperm may be a consequence of the reduced rate of cell expansion rather than the result of an increase in β-glucan synthesis per cell. A model to explain this theory is depicted in Fig. 8. The mechanism we envisage is that deposition of β-glucan around a small cell will result in a thicker cell wall than if the same amount of β-glucan were deposited around a relatively larger cell. Thus, the unusually thick cell walls of *Brachypodium* endosperm cells may be due not to increased synthesis of cell-wall material per cell but to the deposition of a ‘normal’ amount of cell-wall material around an unusually small cell.

In support of this suggested mechanism, *Brachypodium* was found to have both the smallest endosperm cells and the thickest cell walls in a survey of mature grains (Hands *et al.*, 2012). If correct, this mechanism suggests that: (i) reduced cell expansion does not inhibit β-glucan synthesis, and (ii) there is no feedback inhibition from the thickening cell wall per se to the β-glucan synthetic machinery. It also follows that the thickness of the cell wall is determined not only directly by the rate of β-glucan synthesis but also indirectly by the rate of cell expansion.

The model that we have presented in Fig. 8 assumes that there is no difference between barley and *Brachypodium* in the amount of β-glucan synthesized on a per cell basis. However, the reduced rate of starch synthesis in *Brachypodium* may mean that more carbon is available for β-glucan synthesis. Thus, in addition to an indirect interaction between starch and cell-wall synthesis via cell expansion, we cannot rule out an additional direct biochemical link between these biosynthetic pathways at the level of competition for a common sugar nucleotide precursor.

**Evidence of unusual cell-cycle regulation in *Brachypodium* endosperm**

Cell proliferation in *Brachypodium* endosperm appears to be very limited. However, cells in the developing *Brachypodium* endosperm appear to undergo DNA replication (6C endosperm cells with twice the triploid DNA content were observed). This may indicate that some endosperm cells in *Brachypodium* are blocked in the G2 or M phase of mitosis: their DNA is replicated during the S phase, but cell division does not follow. Consistent with unusual cell-cycle regulation, we found severely reduced levels of two cell-cycle-related transcripts, Cyclin A3 and CDKB1, in *Brachypodium* relative to barley. The proteins encoded by these genes are known to be involved in the G2 and M phases in plants (Stals and Inzé, 2001). We also found relatively few, if any, C12 nuclei in *Brachypodium*. This suggests that endoreduplication is reduced in *Brachypodium* endosperm. This may be linked to the block in mitosis described above, as the mitotic and endoreduplication cell cycles are governed, to a large extent, by the same regulatory genes (Sabelli, 2012).

**Summary**

This study revealed that *Brachypodium* grain development differs from that in other members of the Pooideae in several respects. We have provided a mechanism to explain the coordination of cell enlargement with cell-wall thickness and suggest that failure to accumulate starch may explain the lack of cell expansion. Other features such as reduced rates of cell division and endoreduplication may also be connected with the reduction in cell enlargement in *Brachypodium*. Coordination of cell proliferation with cell expansion has been observed in other systems (Dupuy *et al.*, 2010), and it has been suggested that endoreduplication drives grain filling by providing a mechanism for increasing cell size and gene expression (Sugimoto-Shirazu and Roberts, 2003). However, a causal relationship between endoreduplication and cell expansion has been disputed (Leiva-Neto *et al.*, 2004). Modification of sucrose metabolism is also known to have far-reaching effects on grain development, as observed in the maize mutant *miniature1* seed, which lacks cell-wall invertase.
activity (Vilhar et al., 2002). Alternatively, these diverse features may be symptoms of a more fundamental difference between B. distachyon and other Pooidae, for example in the expression of an underlying transcription factor (Hands et al., 2012).

Our work suggests that further study of grain development in B. distachyon, Bromus species, and other Pooidae is likely to shed light on fundamental aspects of storage product synthesis, cell development, and the control of cell-wall synthesis. Grain composition may also impact on ecology, particularly in wild grass species. At present, it is not possible to conclude precisely where the primary site of regulation of grain composition might lie or whether the apparent network of processes might be regulated at multiple points. Nevertheless, our results suggest that the unusual features of grain development and composition in B. distachyon should be taken into account when attempting to use this species as a functional model for the Poaceae.

Supplementary data
Supplementary data are available at JXB online.
Table S1. Sources and glucan content of Pooidae grains.
Table S2. PCR primers.
Table S3. Normalized transcript abundance in developing B. distachyon grains.
Table S4. Normalized transcript abundance in developing barley grains.

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