The deposition and characterization of starch in Brachypodium distachyon

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Abstract

Brachypodium distachyon is a non-domesticated cereal. Nonetheless, Brachypodium was recently introduced as a model plant for temperate cereals. This study compares grain starch metabolism in Brachypodium and barley (Hordeum vulgare). In Brachypodium, we identified and annotated 28 genes involved in starch metabolism and identified important motifs including transit peptides and putative carbohydrate-binding modules (CBMs) of the families CBM20, CBM45, CBM48, and CBM53. Starch content was markedly lower in Brachypodium grains (12%) compared to barley grains (47%). Brachypodium starch granules were doughnut shaped and bimodally distributed into distinct small B-type (2.5–10 µm) and very small C-type (0.5–2.5 µm) granules. Large A-type granules, typical of cereals, were absent. Starch-bound phosphate, important for starch degradation, was 2-fold lower in Brachypodium compared with barley indicating different requirements for starch mobilization. The amylopectin branch profiles were similar and the amylose content was 2-fold lower compared with barley cv. Golden Promise. The crystallinity of Brachypodium starch granules was low (10%) compared to barley (20%) as determined by wide-angle X-ray scattering (WAXS) and molecular disorder was confirmed by differential scanning calorimetry (DSC). The expression profiles in grain for most genes were distinctly different for Brachypodium compared to barley, typically showing earlier decline during the course of development, which can explain the low starch content and differences in starch molecular structure and granule characteristics. High transitory starch levels were observed in leaves of Brachypodium (2.8% after 14 h of light) compared to barley (1.9% after 14 h of light). The data suggest important pre-domesticated features of cereals.

Key words: Barley, Brachypodium distachyon, endosperm, grass domestication, starch biosynthesis, starch granule.

Introduction

Starch is the most important source of carbohydrates for humans, and starch from cereals is of crucial value. Worldwide, starch produced by cereal crops has provided the most important human dietary energy for millennia. In 2012, world production of cereals amounted to 2.3 billion tons (Food and Agriculture Organization, FAO, 2012: http://www.fao.org/docrep/018/a1999e/a1999e.pdf).

Starch is the principal storage product of the majority of plants. It is stored in both photosynthetic and non-photosynthetic organs, among which the grass grain is of central importance, with starch forming its main constituent. Starch is synthesized in well-organised starch granules in amyloplasts in the grain tissue and is made up of two polysaccharides: amylose and amylopectin. Amylose makes up 25–30% of the starch granule, possesses an α-1,4 linkage backbone structure and can be sparsely branched via α-1,6 linkages. Amylose is probably mostly amorphous in the starch granule. Amylopectin typically comprises 70–75% of the starch granule; it is more than 100-fold larger than amylose and contains clustered α-1,6 linkages. Clustering of the amylopectin
chains in the granule results in 9-nm alternating branched amorphous and more-linear crystalline, double-helical lamellae (Damager et al., 2010; Pérez and Bertoft, 2010). Based on X-ray powder diffraction, all normal cereal starch granules show the so-called A-type crystalline polymorph, which distinguishes cereal storage starch from tuberous storage starch, which is often of the B-type crystalline polymorph. Starch is also slightly phosphorylated (0.1–1% phosphate w/w) which acts as a signal for starch remobilization (Blennow and Engelsen, 2010), but interestingly cereal endosperm starch has very low starch-phosphate content (0.01–0.06% phosphate w/w) as compared to tuberous storage starch and transitory leaf starch (Lim et al., 1994; Blennow et al., 2000b, 2002). At the micrometre level, starch assembles into large, distinctly shaped granules of which the form and topography vary tremendously between different plants and organs (Jane et al., 1994).

For dietary needs, large grains are of major importance because grain size is linked to large amounts of its main component, starch, and hence large grains provide superior nutritional quality (Blennow et al., 2013). Nutritional quality has been, and still is, of main importance for human and animal consumption. In the human diet, the dominating importance of grain starch is reflected in the tremendous efforts over millennia to improve starch yield and quality by selection, breeding, and very recently by directed in planta biotechnological modification (Blennow et al., 2013). However, additional quality traits are now demanded that are not linked to high dietary energy, but instead to more complex health-associated traits, such as high dietary fibre and antioxidant content. Many such traits can probably be found in wild progenitors of modern cereals and cereal landraces, making them useful as value-added biopolymers. Knowledge about starch deposition and structure in wild grasses is not extensive, and the genetic capacity for biosynthesis of these new high-value grain components and the composition of ancient and wild grains are insufficiently characterized (Shapter et al., 2008).

It is well known that ancient collected grasses were low yielding and performed suboptimally. The direct human selection of grasses with superior grain led to domestication, but as the initial population that contributed to domesticated plants was small, ‘domestication bottleneck’ effects were unavoidable. This implies loss of genetic diversity and has inevitably resulted in a reduction of genetic factors for potentially important wild traits in domesticated crops compared to their wild ancestors (Doebley, 1989). Likewise, retained alleles have been fixed for desirable traits and these phenotypes became typical across a broad range of domesticated plants. A common suite of these fixed traits is a marker of domestication, known as the ‘domestication syndrome’ (Hammer, 1984; Doebley et al., 2006; Meyer et al., 2012). The suite includes a wealth of traits among which large grains, a non-brittle rachis, local abundance, annuality, shorter seed dormancy, polyphloid, harvestability, and relative ease of seed dehulling have been prioritized (Harlan et al., 1972). The full consequences of the extent of genetic bottlenecks and reduced diversity among domesticated and wild grasses cannot be evaluated based on present knowledge, but wild relatives present an interesting reference system for such investigations. Potentially valuable lost traits can be transferred back into domesticated cereals from wild progenitors.

Brachypodium distachyon (L.) P. Beauv. is a wild grass that has recently been established as a model system for temperate grasses. Brachypodium remnants have been recovered from a ground stone artefact from the Upper Palaeolithic site of Ohalo II in Israel (Piperno et al., 2004), which reveals that this grass was used in human diet while humans were gathering grains for food. However, there is no evidence that Brachypodium has ever been cultivated and finally domesticated. Several traits of Brachypodium such as suboptimal harvestability due to shattering, needle-shaped grains, small plant and grain size, and low-glycemic grain composition support this conclusion that ancestral humans did not consider Brachypodium as a target for domestication. Considering that Brachypodium was the first wild grass with a sequenced genome (Vogel et al., 2010) and developed genetic tool box (Mur et al., 2011), as well as being able to be propagated quickly, it is a valuable model for faster biotechnological screening of novel and undomesticated cereal traits (Bevan et al., 2010). Recent analyses of the Brachypodium grain have revealed features including high protein and (1–3) (1–4)-β-glucan (BG) content (Larré et al., 2010; Guillon et al., 2011; Opanowicz et al., 2011; Hands and Drea, 2012). Such Brachypodium features, together with access to the sequenced genome (Vogel et al., 2010), and developed genetic tools make it a valuable model for further improving modern cereals (Draper et al., 2001; Garvin, 2007; Garvin et al., 2008; Brkljacic et al., 2011; Vain, 2011).

Here we present the first investigation on diversity of starch in grasses. We focussed on the biosynthetic capacity, structure, and composition of starch in the Brachypodium grain. We found distinct differences as well as conserved features in starch biosynthesis capacity, gene expression, and starch structure between Brachypodium and its domesticated relative barley (Hordeum vulgare L. ssp. vulgare). They indicate both important conserved molecular features of starch and its metabolism as well as innovations related to domestication of cereal grain from wild grasses. Specifically, Brachypodium grain displayed low starch content, small granular and amorphous starch granules, and high cell wall and protein content. Distinct features could be linked to specific differences in starch biosynthesis enzyme expression profiles.

Materials and methods

Identification of genes and domain structure

Sequences were collected from the National Centre for Biotechnology Information database (NCBI, http://www.ncbi.nlm.nih.gov/). Sequence similarity searches were performed by BLASTP with standard settings in the Brachypodium genome database (http://www.brachypodium.org/) and in Phytozone (http://www.phytozone.org). Sequences with high percentage identity were selected to represent putative Brachypodium starch enzymes. Correct members of each of the gene classes were confirmed by BLASTP using the NCBI non-redundant database. Additional alignments were done using ClustalW Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/). Alignment and phylogeny relationships were done with MEGA 5
(Tamura et al., 2011). A phylogenetic tree was constructed using the neighbour-joining method. Putative domains were identified using the Pfam database (http://pfam.sanger.ac.uk/). The tandem domain CBM45 was identified manually, using available sequences (Glaring et al., 2011). Signal sequences were identified in Signal 3 (http://www.cbs.dtu.dk/services/SignalP).

Gene expression
Transcriptome data for starch biosynthetic genes for Brachypodium and barley were extracted from previously published data sets obtained from two independent studies: Davidson (2012) and Radchuk (2010), respectively. A Brachypodium gene expression matrix was generated using RNA-Seq Illumina technology, while barley mRNA expression profiles were collected in a cDNA macroarray dataset. Data from barley were unlogged and transformed to relative values (fold change) to allow identification of expression values, and expression trends for starch-related genes in both species were analysed individually.

Plant material
Hordeum vulgare ssp. vulgare cv. Golden Promise (spring barley) and Brachypodium distachyon (L.) P. Beauv. (purple false broom) lines 21 (Bd21) and 21–3 (Bd21-3) were used throughout this study. Bd21 had the first sequenced genome of Brachypodium and Bd21-3 is superior for genetic transformation. Plants were grown in a temperature-controlled greenhouse (22°C day/16°C night) at ambient light levels. Seed samples of B. distachyon were a gift from Dr John Vogel, USDA-ARS, USA.

Starch extraction
Grains were harvested from plants and ground to a fine powder with a tissue lyser (TissueLyser, Retsch, Qiagen). Starch was isolated as described in Carciofi et al. (2011), with additional sieving through a 36 µm mesh. The preparation was inspected by microscopy to evaluate purity and damaged granules and only preparations with less than 1% damaged granules were kept for further analysis.

Extraction of starch granules from leaves was performed as described by Zeeman et al. (1998) with minor modifications. Five-week-old Brachypodium and barley leaves (3 g) were collected after a 10 h light period, immediately frozen in liquid nitrogen and stored at –80°C. Frozen leaves were homogenized using a Polytron PT 3000 Blade-type Homogenizer in 35 ml of 100 mM 3-(N-morpholino) propane sulfonic acid (MOPS), pH 7.2, 5 mM EDTA, and 10% (v/v) glycerol. The homogenate was filtered through two layers of cloth and centrifuged for 10 min, at 4°C (3000 g). The pellet was suspended and washed twice in 30 ml of the same medium including 0.5% (w/v) SDS. A layer on the top of a dense debris pellet was identified as starch granules, which were collected with a pipette, and washed twice with SDS-containing medium and six times with ddH2O to completely remove debris. Sedimented starch was inspected visually for purity and granule quality, and then air dried at room temperature.

Total starch analysis
Mature grains were powdered with a tissue lyser, and the powder was dried by lyophilization overnight; 10 mg samples were used for analysis. Soluble sugars were removed by extraction five times in 80% (v/v) ethanol at 80°C, with intermittent centrifugation at 10 000 × g for 5 min. The powder was suspended in 500 µl of 100 mM Na citrate buffer (pH 4.4) and incubated for 5 min at 80°C to gelatinize the starch. Heat-stable α-amylase (0.1 KNU-T units; Termamyl, Novozymes, Denmark) was added and the samples were incubated for 6 h at 80°C. The tubes were cooled to 60°C and 0.3 AGU units of amyloglucosidase (Dextrozyme, Novozymes, Denmark) were added to samples. Samples were incubated at 60°C overnight, with constant mixing. After centrifugation, the supernatant was analysed for glucose using high-performance anion-exchange chromatography, with a DX 500 system ( Dionex, Sunnyvale, CA, USA) equipped with an S-3500 auto sampler, GP40 pump, ED40 PAD (HPAEC-PAD), and a CarboPac PA-1 column as previously described (Blennow et al., 2000b).

Starch measurements in leaves were performed, with modifications, as described in (Scofield et al., 2009). Brachypodium line 21-3 was used for sampling. Leaf samples were collected over a 14-h light period every 2 h, immediately frozen in liquid nitrogen and stored at –80°C. The tissue was ground to fine powder using a tissue lyser. Samples of 20 mg were used for analysis. Soluble sugars were removed by extraction four subsequent times in 80% (v/v) ethanol at 80°C for 1 h followed with intermittent centrifugations at 13 000 g for 5 min. The powder was suspended in 200 µl of 0.2 M NaOH, incubated for 30 min at 80°C to gelatinize the starch, and neutralized with 15 µl of 1 M HCl. Na acetate buffer (200 µl; 20 mM, pH 4.8) containing α-amylase (0.1 units, Sigma Chemicals) and amyloglucosidase (4 units, Sigma Chemicals) was added and the samples incubated at 50°C overnight with constant mixing. The supernatants were analysed as for the grain samples.

Staining for starch in leaves
Leaves from Brachypodium (line 21-3) and barley were collected after a 10-h light period or a 14-h dark period. Pigments were removed by extraction in 80% (v/v) ethanol at 80°C and the destained leaves were transferred to petri dishes and stained with iodine solution.

Amylose content
The apparent amylose content in starch granules was determined by iodine colorimetry (Wickramasinghe et al., 2009). Each sample (2 mg) was dissolved in 250 µl of 4 M NaOH and incubated for 4 h with gentle mixing. 750 µl of H2O was added to each sample and 10 µl aliquots applied in wells of a 96-well microtiter plate in technical triplicates. 200 µl of diluted iodine solution [stock of 2.6% I2 (w/v), 26% KI (w/v), diluted ×1000 in 1 M HCl] was added, the absorbance measured at 550 nm and 620 nm and amylose concentration calculated by plotting the A620/A550 ratio against standards with known amylose contents (Carciofi et al., 2011).

Amylopectin chain-length distribution
Analysis of the distribution of the amylopectin side chains was performed from starches isolated from grain and leaf tissue of Brachypodium and barley. Commercially available grain starch from rice (Oryza sativa L. ssp. indica) was used as a control. Each sample (1 mg) was incubated with 1 M NaOH at 80°C for 5 min and the pH was neutralized with 10 µl 1 M HCl. Citrate buffer (100 µl; 0.1 M) was added containing 1 µl of isoamylase (Sigma Chemicals), samples were incubated for 1 h at 40°C, shortly centrifuged, and supernatants were analysed by high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) using a CarboPac PA-100 column as described previously (Blennow et al., 2000b).

Differential scanning calorimetry
Starch granule thermal characteristics in water were analysed using differential scanning calorimetry (DSC) as described previously (Carciofi et al., 2011) using a Perkin Elmer Diamond DSC operated from 30°C to 100°C at a scanning rate of 10 °C min⁻¹. The starches extracted from endosperm were analysed in slurries of 3 mg native starch and 12 µl 10 mM NaCl in technical triplicates. The endotherm transition was identified and the Perkin Elmer Pyris 7.0 software used to determine the parameters onset temperature (T0), peak temperature (Tp), conclusion temperature (Tc), and enthalpy transition energy (∆H).
X-ray diffraction
X-ray diffraction (XRD) analyses were performed as described previously (Tawil et al., 2011).

Glucose-6-phosphate and glucose-3-phosphate content
The degree of starch phosphorylation was determined as the content of glucose-6-phosphate (Glc-6-P) and glucose-3-phosphate (Glc-3-P) after complete starch hydrolysis as described previously (Blennow et al., 1998) using an HPAEC-PAD system fitted with a CarboPac PA-1 column. Concentrations were determined using standard potato starches with known Glc-6-P and Glc-3-P content as determined by NMR (Blennow et al., 2000a, 2000b).

Scanning electron microscopy
Grains were sectioned (400 μm) and dry starch granules previously purified from Brachypodium and barley grains were mounted on carbon tabs on aluminium stubs. Samples were sputter coated with gold/palladium for 120 seconds and observed with a secondary detector at an accelerating voltage of 10 kV in Quanta 200 scanning electron microscopy (SEM; FEI Company, Eindhoven, Netherlands).

Bright field and polarised light microscopy
Sections of 400 μm thickness of both Brachypodium and barley grains were produced using a microtome with vibrating blade Microm HM 650 V-Thermo Scientific. The slices were stained with iodine solution (as above) and observed with light microscopy using a Leica DMR HC fluorescence microscope combined with digital camera Leica DC 300F. Shape and size of extracted starch granules from seeds and leaves were investigated using bright field (BF) microscopy after suspending powder in iodine solution. Granule birefringence was investigated using a cross-polarizer.

Confocal laser scanning microscopy
Native starch granules were stained with 8-amino-1,3,6-pyrenesulfonic acid (APTS) and afterwards confocal laser scanning microscopy (CLSM) was performed as described previously (Blennow et al., 2003).

Starch particle size analysis
For Brachypodium, 10 light micrographs of 600 iodine-stained starch granules were recorded and the granules were measured using ImageJ software (Schneider et al., 2012). The size distribution for barley starch was measured by laser diffraction using a Microtrac S3000 analyzer (Microtrac, PA, USA). Starch granules (50mg) were suspended in ddH₂O and sonicated for 10 min before analysis to avoid aggregation. The data was analysed using Microtrac Flex Software. For both Brachypodium and barley, granule size distribution was calculated on the basis of number and volume frequency.

(1–3)(1–4)-β-glucan content
(1–3)(1–4)-β-glucan analysis was performed according to Guillou et al. (2011) using a Megazyme mixed-linkage β-glucan kit (AOAC method 996.11; (1–3)(1–4)-β-glucan, AOAC method 995.16) with the modification that quantification of glucose was done using HPAEC-PAD chromatography (Blennow et al., 2000b).

Total nitrogen and protein
Total nitrogen and carbon contents of the grains were determined on finely ground grains using an ANCA-SL/GSL elemental analyser (SerCon, UK) attached to a stable isotope mass spectrometer. A nitrogen-to-protein conversion factor of 5.7 was used for protein content (Mosse, 1990).

Results
Brachypodium possesses full genetic capacity for starch biosynthesis
The comparative genetic capacity for starch biosynthesis and degradation in Brachypodium and barley was investigated in silico and the enzyme functional gene structure deduced. We identified 23 unique genes and annotated them in the groups starch synthases (SSs), starch-branching enzymes (SBEs), debranching enzymes (DBEs), α-glucan phosphorylase (PHOs), and glucan water dikinases (GWDs) according to their catalytic domains. Recent annotations (Li et al., 2012; Trafford et al., 2013) were confirmed with updates for BdAPL1, BdSSIIIa, BdSBEIII, ISAIII, and BdGWDs, while ADP-glucose pyrophosphorylase (BdAGPase) genes were identified previously (Comparot-Moss and Denyer, 2009). GWDs were included to provide a comprehensive comparative list of Brachypodium and barley genes for starch metabolism (Table 1). The number of identified paralogues for DBEs, GWDs, SBEs and PHOs were identical for Brachypodium and barley. Brachypodium had additional SSII and AGP-L paralogues. These results demonstrate that the genetic capacity for starch metabolism is largely conserved through grass evolution and domestication.

In order to identify the main starch biosynthetic relationships in Brachypodium compared with major members of the Poaceae family and selected well-characterized dicotyledonous plants we constructed a phylogenetic tree with these members based on the starch synthase protein sequences (Supplementary Figure S1). Each clade turned out to constitute a cluster with specific enzymatic function, experimentally documented for many members. The putative Brachypodium BdGBSSIb sequence is an outlier. Its sequence codes for a catalytic domain, but the protein sequence is possibly truncated indicating that it might be a pseudogene.

The functional domain structure of transferase orthologues are expected to be well conserved across the Poaceae family and we therefore identified the SS catalytic domain, non-catalytic carbohydrate-binding module (CBM) with affinity to starch and plastid-targeting signal peptides (Fig. 1). Four different CBMs were identified and positioned in the sequences belonging to the CBM family CBM20 (Christiansen et al., 2000). In SSIII three internal repeats of CBM53 were present in the middle part of the coding sequence. These domains have been suggested to have a regulatory role (Valdez et al., 2008). CBM45 is present as a tandem repeat domain present in GWD1 and GWD2 (Glaring et al., 2011) and these are indicated to determine the lengths of the chains to be phosphorylated (Mikkelsen et al., 2006). CBM48, typical for SBEs, was identified in BdSBEs, and CBM20 was found in the BdPWD/GWD3 (Christiansen et al., 2009a).

Brachypodium and barley starch genes have different gene expression patterns
Comparative expression profiling of genes involved in starch biosynthesis in Brachypodium and barley was carried out based on available data (Radchuk et al., 2009; Davidson...
Table 1. Comparative list of the in silico predicted starch biosynthesis and degradation proteins in Brachypodium and barley genomes

| Enzyme name and abbreviation | Purple false brome | Barley | Reference for barley |
|------------------------------|--------------------|--------|----------------------|
| ADP-glucose pyrophosphorylase  | Small subunit      |        |                      |
| AGPase (EC 2.7.7.27)         | AGP-S              | AGP-S1 cyt | Radchuk et al. (2009); Huang et al. (2011) Comparot-Moss and Denyer (2009) |
|                             | BdAPS1 cyt         | CAA88449 | Radchuk et al. (2009); Huang et al. (2011); Comparot-Moss and Denyer (2009) |
|                             | BdAPS2             | AGP-S2  | Huang et al. (2011)   |
|                             | Bradi3g22330       | AAO16183 | Huang et al. (2011)   |
|                             | Bradi4g27570       | AGP-S3  | Huang et al. (2011)   |
|                             | Bradi4g53500       | CAXS1552| Huang et al. (2011)   |
|                             | AGP-L              | AGP-L1  | Huang et al. (2011)   |
|                             | Bradi1g09537       | CAA47626| Huang et al. (2011)   |
|                             | Bradi2g14970       | AGP-L2  | Huang et al. (2011)   |
|                             | Bradi2g14970       | AAC49729| Huang et al. (2011)   |
| Starch synthase (EC 2.4.1.21) | Granule-bound starch synthase | GBSSI   | Radchuk et al. (2009) |
|                             | GBSSI              | GBSSIa  | Radchuk et al. (2009) |
|                             | Bradi1g50090       | GBSSIb  | Radchuk et al. (2009) |
|                             | GBSSIi             | GBSSIb  | Radchuk et al. (2009) |
|                             | Bradi2g41590       | GBSSIb  | Radchuk et al. (2009) |
|                             | Bradi4g0650        | GBSSIb  | Radchuk et al. (2009) |
| Soluble starch synthase     | SSI                | SSI     | Radchuk et al. (2009) |
|                             | Bradi1g48610       | SSI     | Radchuk et al. (2009) |
|                             | SSIIa              | SSIIa   | Li et al. (2003)      |
|                             | Bradi1g45130       | SSIIc   | Yan et al. (2009)     |
|                             | SSIIb              | SSIIc   | Yan et al. (2009)     |
|                             | Bradi3g59440       | AK251488| Yan et al. (2009)     |
|                             | SSIIc              |         |                      |
|                             | Bradi3g27260       |         |                      |
|                             | SSIIa              | SSIIa   | Radchuk et al. (2009) |
|                             | Bradi3g15027       | SSIIb   | Radchuk et al. (2009) |
|                             | SSIIb              | SSIIb   | Radchuk et al. (2009) |
|                             | Bradi5g22310       | SSIV    | Radchuk et al. (2009) |
|                             | SSIV               | SSIV    | Radchuk et al. (2009) |
|                             | Bradi2g18810       | SSIV    | Radchuk et al. (2009) |
| Starch-branching enzyme (EC 2.4.1.18) | SBEI               | SBEI    | Radchuk et al. (2009) |
|                             | SBEI               | SBEI    | Radchuk et al. (2009) |
|                             | Bradi1g29850       | SBEI    | Radchuk et al. (2009); Hazard et al. (2012) |
|                             | SBEII              | SBEII   | Radchuk et al. (2009); Hazard et al. (2012) |
|                             | SBEII              | SBEII   | Radchuk et al. (2009); Hazard et al. (2012) |
|                             | Bradi5g09170       | SBEIIb  | Radchuk et al. (2009); Hazard et al. (2012) |
|                             | SBEIIb             | SBEIIb  | Radchuk et al. (2009); Hazard et al. (2012) |
|                             | Bradi3g44760       | SBEIIIb | Radchuk et al. (2009); Hazard et al. (2012) |
|                             | SBEIIIb            | SBEIIIb | Radchuk et al. (2009); Hazard et al. (2012) |
|                             | Bradi1g41970       | SBEIIIb | Radchuk et al. (2009); Hazard et al. (2012) |
| Starch-debranching enzyme   | Isoamylase         | ISA     | Radchuk et al. (2009) |
| (EC 3.2.1.68)               | ISA                | ISA     | Radchuk et al. (2009) |
|                             | ISA1               | ISA1    | Radchuk et al. (2009) |
|                             | Bradi3g40410       | ISA1    | Radchuk et al. (2009) |
|                             | ISA2               | ISA2    | Radchuk et al. (2009) |
|                             | Bradi2g62170       | ISA2    | Radchuk et al. (2009) |
|                             | ISA3               | ISA3    | Radchuk et al. (2009) |
|                             | Bradi4g32707       | ISA3    | Radchuk et al. (2009) |
| Limit dextrinase            | PUL                | PUL     | Radchuk et al. (2009) |
|                             | PUL                | PUL     | Radchuk et al. (2009) |
|                             | Bradi5g0540        | PUL     | Radchuk et al. (2009) |
| α-Glucan phosphorylase (2.4.1.1) | PHO                | PHO1    | Radchuk et al. (2009) |
|                             | PHO1               | PHO1    | Radchuk et al. (2009) |
|                             | Bradi1g08070       | PHO1    | Radchuk et al. (2009) |
|                             | PHO2               | PHO2    | Radchuk et al. (2009) |
|                             | Bradi2g55120       | PHO2    | Radchuk et al. (2009) |

*Reference for barley indicates the source of the barley sequences.*
Brachypodium starch is branched as is typical for temperate grasses, but granules are distinct

In order to study the molecular composition of the starch of Brachypodium, amylose, starch-phosphate (Table 2), and amylpectin chain-length distribution (Fig. 2) were analysed in pure starch. Moreover, since molecular structure probably affects starch granule organization, the starch granule size distribution, morphology, topography, and crystallinity were investigated.

The amount of amylose in Brachypodium starch (34%) was higher compared to the level found in barley starch (29%). On the other hand, Brachypodium starch contained 2-fold less phosphate (0.35–0.40 nmole Glc-6-P mg⁻¹ and 0.12–0.15 nmole Glc-3-P mg⁻¹) as compared to barley (0.80 nmole Glc-6-P mg⁻¹ and 0.32 nmole Glc-3-P mg⁻¹).

The amylpectin chain-length distribution, as compared to rice and barley (Carciofi et al., 2012) (Fig. 2) was analysed. These distributions are fully consistent with those generally found in the plant kingdom (Blennow et al., 2000b). However, interesting fingerprints specific for Brachypodium and barley were found in the DP 14–20 region where both Brachypodium and barley had a distinct peak at DP 19–20, which was lacking in the rice distribution.

The shape and size of Brachypodium starch granules as studied by light microscopy and SEM were examined and compared to barley granules (Fig. 3A, B). Light microscopy was used to study granule shape, while SEM gave more insight about the topography and surface of starch granules (Fig. 3C, D). The shapes of the Brachypodium granules were
round to ellipsoid, but many were flattened, appearing as concave disks with depressions in the centre forming doughnut-shaped structures. Their surfaces were smooth, just like barley granules. Pores on the surfaces of *Brachypodium* granules could not be observed, suggesting that they do not exist or are too small to be seen. In the *Brachypodium* grain, we
confirmed that starch was exclusively found in the endosperm cells (Fig. 3G, H) (Opanowicz et al., 2011; Trafford et al., 2013).

Anisotropic orientation of glucan chains in the starch granule, as deduced from polarization microscopy of Brachypodium grain starch granules (Fig. 3E, F), shows birefringence indicating that the molecular orientation was probably radial (Glaring et al., 2006). The majority of starch granules of Brachypodium only showed birefringence in the peripheral 1–3 µm layer, indicating lower levels of orientation of polymer chains in the central granule region than barley starch granules which show clear birefringence.

The internal structures of the Brachypodium starch granules were investigated using CLSM (Fig. 4A–F) giving a more comprehensive insight into the internal microstructure of the grain storage starch granules. A discoid shape and depressions were clearly visible in the granules of all sizes, but pores, channels, or growth rings were not evident. The fluorescent probe APTS is linked to the reducing ends of each polysaccharide and hence amylose, being considerably smaller, generated the most fluorescence. White arrows on the micrographs indicate amylose-rich areas of Brachypodium granules. The inner structure of Brachypodium granules was similar to the structure of barley granules (Shaik et al., manuscript in preparation); even if the outer shape was different, barley granules were more oval and filled, while Brachypodium granules kept the doughnut-shape.

As measured by image analysis, the estimated size of the Brachypodium endosperm starch granules ranged between 0.5 and 12 µm in Brachypodium, displaying a bimodal size

Table 2. Grain and starch granule composition in two lines of Brachypodium and barley

|                      | Bd 21  | Bd 21-3 | Barley GP |
|----------------------|--------|---------|-----------|
| **Grain composition**|        |         |           |
| Protein content (%)  | 28.57  | 28.53   | 12.26     |
| Total starch content (%) | 12.30 ± 0.87 | 11.10 ± 2.27 | 47.10 ± 0.83 |
| Mixed beta glucan content (%) | 29.30 ± 0.77 | 32.20 ± 1.71 | 4.10 ± 0.84 |
| **Starch granule composition** |        |         |           |
| Starch amylose content (%) | 34.0 ± 1.8 | 34.5 ± 1.0 | 29.9<sup>c</sup> |
| Starch phosphate content (nmol Glc-6-P mg<sup>-1</sup>) | 0.40 ± 0.05 | 0.35 ± 0.03 | 0.81 ± 0.37<sup>b</sup> |
| Starch phosphate content (nmol Glc-3-P mg<sup>-1</sup>) | 0.15 ± 0.02 | 0.12 ± 0.01 | 0.32<sup>b</sup> |

<sup>a</sup> SD of at least triplicates is indicated. <sup>b</sup> Carciofi et al. (2011). <sup>c</sup> Carciofi et al. (2012).

Fig. 2. Chain-length distribution of amyllopectin from Brachypodium lines 21 and 21-3, barley, and rice. Arrows indicate peaks at DP 14, DP 19-20, and DP 45.
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distribution (Fig. 5). The two classes are small (2.5–10 μm) granules and very small (0.5–2.5 μm) granules, which according to Bechtel (1990) would correspond to B- and C-type granules. Seemingly, *Brachypodium* did not possess starch granules comparable to large A-type granules in barley. Starch granules in barley showed a typical bimodal distribution consisting of larger ≥15 μm A-type granules and smaller 2–5 μm B-type granules (Fig. 5) (Jane et al., 1994).

The crystalline polymorph and degree of crystallinity were investigated for isolated *Brachypodium* and barley starch granules by powder X-ray crystallography (Fig. 3I). *Brachypodium* starch showed an A-type crystalline polymorph (diffraction peaks at 2θ at 15.1°, 17.8°, and 23°), which is typical for cereal storage starch. However, the wide-angle X-ray scattering (WAXS) diagram of *Brachypodium* contains traces of V-type crystalline polymorph (diffraction peaks at 2θ at 7°, 13°, and 20°). The degree of crystallinity was low and estimated to be 10%, which is two times lower than that observed for barley starch (20%) (Carciofi et al., 2012).

Crystalline packing as analysed by dissolution in aqueous medium analysed by DSC revealed that the enthalpy of melting of *Brachypodium* starch was 2.72 and 3.10 J g⁻¹ for Bd21 and Bd21-3, respectively and 6.32 J g⁻¹ for barley (Table 3), supporting the more disordered structure of the *Brachypodium* starch granules as compared to barley starch granules.

**Transient starch in Brachypodium is higher than in barley**

The starch content was measured in leaves of *Brachypodium* and barley over a 12-h light period, from 2 h to 14 h of light exposure, every 2 h. The rate of starch biosynthesis progressed virtually linearly after 14 h of light, reaching 2.9%. Interestingly, the amount of starch in barley also progressed linearly, but more steeply. After an 8-h light period, deposition of starch ceased and resulted in a lower amount (2%) than in *Brachypodium* after a 14-h light regime (Fig. 6A). Iodine staining of leaves showed a slightly darker colour in *Brachypodium* as compared to barley, confirming a higher concentration of starch in *Brachypodium* (Fig. 6B), which is in accordance with the measured amount of starch after 10 h of light. Comparative amylopectin chain-length distribution analysis of leaf starch from *Brachypodium* and barley showed no significant difference at the end of the 14-h light period (data not shown). The *Brachypodium* leaf starch granule shape, size, and topography were very similar to that of barley leaf starch, having small, flattened, and round-shaped morphology as deduced from SEM (Fig. 6C, D).

**Discussion**

Our study comprises a thorough characterization of biosynthesis and deposited structures of starch in the grass *Brachypodium*. *Brachypodium* possesses all the genes required for starch synthesis. Important starch-binding CBM motifs are conserved. Hence, any differences found in starch content
and structure are likely to be found at transcriptional or post-translational levels. Gene-expression dynamics cannot be directly translated into enzyme activity in the cell. However, such data provide the general long-term capacity for specific activities to be interpreted in terms of starch molecular structure. For *Brachypodium*, gene expression was generally low throughout the endosperm including the expression of GWD starch phosphorylase. Moreover, distinct patterns in gene expression of *Brachypodium* and barley were found and can possibly explain the lower starch biosynthesis. As an example, the low expression levels found for ssI and sbeI substantiates this view (Trafford et al., 2013).

**Table 3.** Thermal properties for two lines of *Brachypodium* and barley: the melting enthalpy ($\Delta H$), onset ($T_o$), peak ($T_p$), and conclusion temperature ($T_c$)$^a$

|          | Bd 21  | Bd 21-3 | Barley GP$^b$ |
|----------|--------|---------|---------------|
| $\Delta H$ (J g$^{-1}$) | 2.7 ± 0.2 | 3.1 ± 0.1 | 6.3 ± 0.2 |
| $T_o$ (°C)     | 54.5 ± 0.8 | 54.0 ± 0.2 | 61.4 ± 0.4 |
| $T_p$ (°C)     | 59.9 ± 0.1 | 59.8 ± 0.2 | 66.0 ± 0.1 |
| $T_c$ (°C)     | 67.6 ± 0.1 | 65.4 ± 0.1 | 70.1 ± 0.0 |

$^a$ SD of at least triplicates is indicated. $^b$ Carciofi et al. (2011).
Some peculiarities found for *Brachypodium* including small size, more flat shapes, absence of large A-type granules, low crystallinity, higher molecular disorder, and isotropy compared to barley starch granules demonstrate profound differences in their biosynthesis. Interestingly *Brachypodium* starch granules show a high level of similarity to immature cereal granules ([Borén et al., 2008](#)), suggesting that starch biosynthesis in wild grasses as compared to domesticated cereals is slower or even arrested before granules adopt true crystalline lamellae and the layers typical for cereal starch granules. The very small (under 2.5 µm) and small (2.5 to 10 µm) granule populations cannot directly be translated to the A-, B-, and C-type classification as typically done according to the size, shape, and timing of their initiation in the endosperm ([e.g. Bechtel et al., 1990; Stamova et al., 2009](#)). The morphology of *Brachypodium* granules as identified in this study is unique.

Fig. 6. (A) Changes of starch content in leaves (DW) in *Brachypodium* line 21-3 (solid line) and barley (dashed line) during the light regime. (B) Leaves of *Brachypodium* and barley stained with iodine after a 24-h night (n) and a 10-h day (d). Shape and size of starch granules extracted from leaves of (C) *Brachypodium* and (D) barley as visualized with SEM. SD of at least triplicates is indicated. This figure is available in colour at *JXB* online.
and hence difficult to directly compare to spheroid- and lens-shaped granules typically found in starches in comparable species. In our study, only size was used to discriminate starch granule type and it remains to be shown whether the B- and C-type classification holds in the sense of development of the grain and biosynthetic mechanism. In wheat (Yin et al., 2012) the larger A-type granules first appear at 3 DAP, while smaller B-type granules occur around 15 DAP and both grow in size until the grain matures. There have been indications of B-granules being synthesized in connection to, and dependent on, the presence of A-type granules (e.g. Glaring et al., 2006). In Brachypodium, the C-type granules might originate by budding from the B-type granules, but this mechanism remains to be confirmed. Hence, prolonged granule maturation and the formation of second generations of granules in the shape of small B- or C-type granules do not seem to be domestication effects. B-type starch granules are present in the genus Hordeum (Baum and Bailey, 1987) as well as in most species of wild wheat (genus Aegilops), where a genetic marker for B-type granules was identified (Stoddard and Sarker, 2000; Howard et al., 2011) indicating that the biosynthesis of B-type granules is not necessarily linked to domestication events.

In summary, the differences in starch granule size and morphology in Brachypodium as compared to barley may occur for many reasons. Main differences were found in developmental maturity of the starch granules, the slow starch biosynthesis linked to low expression levels, and the different expression profiles of genes. Different expression profiles can potentially result in dissimilar balance of enzyme activities. A completely different starch biosynthetic mechanism is unlikely because of the conserved features of the enzymes expressed.

The substantially different grain compositions of the Brachypodium grain as compared to barley grain – mainly lower levels of starch and higher levels of protein and BG in Brachypodium – support recent data (Larre et al., 2010, Guillon et al., 2011, Trafford et al., 2013) even though the actual levels differ indicating that Brachypodium grain composition, as compared to the more robust barley, is subject to genotypic and/or conditional variation. The data also support the general notion (Blennow et al., 2013; Fig. 2) that domestication has driven an increase in endospermic starch biosynthesis at the expense of protein, cell wall BG, and possibly other constituents resulting in high content of dietary and easily accessible carbohydrate. The cell walls in Brachypodium endosperm are tremendously thick (Fig. 4G, H) in agreement with its high BG content, and are far too stiff for food and feed purposes. Interestingly, the high levels of BG provide a grain high in dietary fibre, an important asset for modern food. As indicated (Trafford et al., 2013) the increased BG:starch ratio can be a direct consequence of reduced activity of Brachypodium enzymes in starch biosynthesis as compared to domesticated barley.

In leaves of Brachypodium, starch granules were of the same size as for barley. Interestingly, the rate of starch synthesis in Brachypodium and barley was different, resulting in higher leaf starch content in Brachypodium. Fructans are the major storage carbohydrate in the vegetative tissues of grasses, including barley (Hendry, 1993). We cannot exclude a similar situation in Brachypodium, even though our results indicate that leaf starch in Brachypodium is of greater importance as an energy reserve than in barley. This indicates that leaf starch in Brachypodium can be of greater importance as an energy reserve to be utilized in varying growth conditions and climates in this wild grass than in barley. In conclusion, more thorough cross-species analysis of wild-grass grains is required to assess the potential of wild grasses like Brachypodium as genetic resources to introduce novel high-value traits into modern cereals.

Supplementary material

Supplementary data can be found at JXB online.

Supplementary Figure S1. Phylogenetic tree based on predicted protein sequences of different starch synthases.

Supplementary Figure S2. Comparison of expression profiles of selected starch biosynthesis genes in Brachypodium and barley in developing endosperm.

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