RESEARCH PAPER

Overexpression of HvCslF6 in barley grain alters carbohydrate partitioning plus transfer tissue and endosperm development

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Abstract

In cereal grain, sucrose is converted into storage carbohydrates: mainly starch, fructan, and mixed-linkage (1,3;1,4)-β-glucan (MLG). Previously, endosperm-specific overexpression of the HvCslF6 gene in hull-less barley was shown to result in high MLG and low starch content in mature grains. Morphological changes included inwardly elongated aleurone cells, irregular cell shapes of peripheral endosperm, and smaller starch granules of starchy endosperm. Here we explored the physiological basis for these defects by investigating how changes in carbohydrate composition of developing grain impact mature grain morphology. Augmented MLG coincided with increased levels of soluble carbohydrates in the cavity and endosperm at the storage phase. Transcript levels of genes relating to cell wall, starch, sucrose, and fructan metabolism were perturbed in all tissues. The cell walls of endosperm transfer cells (ETCs) in transgenic grain were thinner and showed reduced mannan labelling relative to the wild type. At the early storage phase, ruptures of the non-uniformly developed ETCs and disorganization of adjacent endosperm cells were observed. Soluble sugars accumulated in the developing grain cavity, suggesting a disturbance of carbohydrate flow from the cavity towards the endosperm, resulting in a shrunken mature grain phenotype. Our findings demonstrate the importance of regulating carbohydrate partitioning in maintenance of grain cellularization and filling processes.

Keywords: Barley, carbohydrate partitioning, cavity, fructan, (1,3;1,4)-β-glucan, grain morphology, starch, sucrose.

Abbreviations: DAP, days after pollination; DP, degree of polymerization; ETC, endosperm transfer cell; 1-FEH, fructan 1-exohydrolase; 6-FEH, fructan 6-exohydrolase; 1-FFT, fructan:fructan-1-fructosyltransferase; 6G-FFT, fructan:fructan-6G-fructosyltransferase; GBSS, granule-bound starch synthase; HPAEC–PAD, high pH anion exchange chromatography with pulsed amperometric detection; HvCslF6, cellulose synthase-like gene F6; HvCslH1, cellulose synthase-like gene H1; HvCslJ, cellulose synthase-like gene J; ISO, isoamylase; LD, limit dextrinase; MALDI-MSI, matrix-assisted laser desorption/ionization mass spectrometry imaging; MLG, mixed-linkage (1,3;1,4)-β-glucan; NP, nucellar projection; pAsGlo1, oat globulin AsGlo1 promoter sequence; SBE, starch branching enzyme; 6-SFT, sucrose:fructan-6-fructosyltransferase; SS, starch synthase; 1-SST, sucrose:sucrose-1-fructosyltransferase WT, wild type; WT(tc), wild type regenerated from tissue culture.

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Introduction

The major storage carbohydrates in cereal grains include starch, fructan, and mixed linkage (1,3;1,4)-β-glucan (MLG). In Hordeum vulgare L. (barley) grain, starch represents the dominant storage polysaccharide contributing between 52% and 72% of the dry weight (Henry, 1988). In contrast, fructan constitutes 1–4% and MLG 4–10% of the total dry weight (Åman et al., 1985; Henry and Saini, 1989; Burton and Fincher, 2012; Nemeth et al., 2014). Changing carbohydrate metabolic pathways significantly influences grain development and final composition, and thus represents a key factor for future development of barley varieties with application-specific grain quality. In addition, the need to lower processing costs and reduce wastage of valuable nutrients, such as nutrients lost during pearling, have resulted in greater interest in hull-less barley varieties.

Allocation of carbohydrate occurs as soon as the grain begins to form and continues throughout development. At the pre-storage phase [0–6 days after pollination (DAP)], the maternal pericarp, besides its own chloroplast activities, receives additional sucrose from vascular tissue; this sucrose undergoes conversion to hexose sugars which are then used for transient starch reserves (Radchuk et al., 2009; Sreenivasulu et al., 2010). Concurrently, the maternal nucellus begins to disintegrate partly to support cell division and cellularization events in the early endosperm at 3 DAP (Sreenivasulu et al., 2010; Domínguez and Cejudo, 2014). MLG is first deposited in the earliest cell walls at 4–5 DAP (Kohorn et al., 2006), and oligofructans are detected in the vacuoles (Wagner and Wiemken, 1986). From 6 to 8 DAP, genes related to energy production and storage are expressed (Wobus et al., 2005; Sreenivasulu et al., 2010). Maintaining cytosolic sucrose homeostasis is indispensable for correct starch synthesis. Starch storage in the endosperm begins at 9 DAP (Olsen, 2001; Sreenivasulu et al., 2010), coinciding with a high sucrose to hexose ratio (Wobus et al., 2005).

Starch biosynthesis enzymes include granule-bound starch synthase (GBSS), starch synthase (SS), isoamylase (ISO), limit dextrinase (LD), starch branching enzyme (SBE), and ADP-glucose pyrophosphorylase (Radchuk et al., 2009; Jeon et al., 2010; Keeling and Myers, 2010). Fructans are synthesized by sucrose:1-fructosyltransferase (1-SST), fructan:fructan-1-fructosyltransferase (1-FFT), sucrose:fructan-6-fructosyltransferase (6-SFT), and fructan:fructan-6G-fructosyltransferase (6G-FFT). Hydrolysis of fructans is mediated by fructan 1-exohydrolase (1-FEH) (Vijn and Smeekens, 1999). MLG accounts for 70% of cell wall material in barley endosperm (Fincher, 1975; Guillou et al., 2011; Burton and Fincher, 2014), and MLG synthesis requires cellulose synthase-like enzymes HvCslF6, HvCslH1, and HvCslJ (Doblin et al., 2008; Burton et al., 2011; Little et al., 2018) in balance with (1,3;1,4)-β-glucanase (Hrmova and Fincher, 2001).

Manipulation of genes associated with these pathways is a common strategy to produce crops with the desired grain composition. This manipulation can have a significant influence on grain development and composition. For example, the M292 mutation in the starch synthase IIa (SSIIa) gene produced a shrunken grain phenotype, with a reduction in starch content, coinciding with significantly increased sucrose, fructan, and MLG content (Clarke et al., 2008). The same phenotype was reported for low-starch barley lines with RNAi suppression of all starch branching enzyme genes (Carciofi et al., 2012). It has been suggested that reduced metabolic uptake of sucrose in the endosperm transfer cell (ETC) region can cause an excessive accumulation of fluid that enlarges the grain cavity, resulting in shrunken grain after desiccation (Carciofi et al., 2012; Shaik et al., 2016). Larger grain cavities have previously been observed in barley lines overexpressing HvCol6F6, and grain from these lines had higher MLG and reduced starch content. Morphological changes in these lines were detected in aleurone tissues that were on average three cell layers thick, with strongly defined walls and a pronounced cubic shape as well as deformed and, in places, completely missing transfer cells at the grain-filling phase (15 DAP). Mature transgenic grain had reduced size relative to control lines, a shrunken and darkened appearance, and increased brittleness (Lim et al., 2018). In this study, we used transgenic grains overexpressing the HvCol6F6 gene as a model system to investigate the effects of increased MLG on carbohydrate metabolism, cavity formation, and cell identity during grain development.

Materials and methods

Plant materials

Barley plants were grown following Lim et al. (2018) under a day/night temperature regime of 23 °C/15 °C. Progeny of the T1 generation from four independent transformed lines (15–3, 18–6, 25–5, and 16–5) overexpressing the HvCol6F6 gene were selected; these lines were referred to as lines F6-15, F6-18, F6-25, and F6-16 in Lim et al. (2018); in addition, Torrens [wild type (WT)] and WT plants regenerated from tissue culture [WT(tc)] were selected and analysed. Developing grains from individual plants were collected from the middle of the spike from 7 to 24 DAP at approximately mid-day, covering all phases of grain development. Mature grain was also collected. The ‘outer tissues’, endosperm, and embryo samples were separated using a scalpel and fine forceps, snap-frozen in liquid nitrogen, and kept at −80 °C until required. Experiments were performed on three biological replicates, consisting of at least 10 grains per replicate, with two technical replicates each, except where noted in the figure legend.

Vector construction and Agrobacterium-mediated barley transformation of pAsGlo1:3xnisYFP

DNA fragments were synthesized by Genscript (USA) and inserted into the pUC57 vector. The 2.6 kb 3xnisYFP gene (MA044) was adapted from Ueda et al. (2011) and codon optimized for barley using the online tool at https://sg.idtdna.com/CodonOpt (accessed 1 October 2019). The sequence for the 994 bp oat globulin AsGlo1 promoter sequence (pAsGlo1; MA003) was obtained from Vickers et al. (2006) and modified to include 5’-HindIII and 3’-KpnI restriction sites. The HindIII/KpnI pAsGlo1 fragment from MA003 was excised and inserted into the Gateway-compatible pMD32 vector (Curtis and Grossniklaus, 2003) in place of the double 35S promoter, creating MA009. The 3xnisYFP sequence was amplified from pUC57 using pUC57-attL1_FWD (5’-ACCTCGGGAATGCATCTTAGATCA-3’) and SacI-attL2_REV (5’-CAAATGTGTTTATTTGACTGATGTTGCAGTGTCGTTGCCAACCATTGATGACAAATTTTTTATATATATTGCAACCAATTTTACGTGGTCACATATTGGAACGTCT-3’) primers and HiFi Platinum Taq (Thermo Fisher Scientific), resulting in a PCR fragment flanked by attL1 and attL2 Gateway-compatible sites. The
The amount of nitrogen in mature grain samples was analysed using the Dumas combustion method following Jung et al. (2003). Protein was calculated from the nitrogen content of the material, using a nitrogen conversion factor of 6.25.

RNA isolation and cDNA synthesis

Total RNA was extracted from all tissue homogenates using an Invitrogen™ TRIzol™ (Thermo Fisher Scientific), DNA was removed using a DNA-free kit following the manufacturer's instructions (Ambion™, Thermo Fisher Scientific), and was used as the template for cDNA synthesis following Burton et al. (2008).

Real-time quantitative PCR (qPCR)

Gene-specific primers were designed from the 3'-untranslated regions of full-length cDNAs. Amplification was achieved using the gene-specific primers purified using HPLC following Burton et al. (2008) and sequenced at the Australian Genome Research Facility, Adelaide, South Australia. PCR primer sequences are provided in Supplementary Table S2 at JXB online. qPCR analysis was carried out following Burton et al. (2008). Heatmap representation was generated using Multiple Experiment Viewer (MeV) software (http://mev.tm4.org/) accessed 1 October 2019.

Protein extraction, SDS-PAGE, and immunoblotting

Microsomes from frozen barley grain tissues (10 grains each) were isolated according to Song et al. (2015). Protein concentration was measured using the BCA protein assay kit (Thermo Fisher Scientific). Protein gel electrophoresis was performed with 30 µg of protein using the NuPAGE Bis-Tris electrophoresis system (Invitrogen) on 4–12% gradient gels (Novex, Life Technologies) with the molecular mass marker Precision Plus Protein Kaleidoscope (BioRad). After electrophoresis, proteins were transferred onto nitrocellulose membrane (Nitrobind, 0.22 µm, Thermo Fisher Scientific) using the XCell II Blot Module (Invitrogen) according to the manufacturer's protocol and processed according to Doblin et al. (2009). The membrane was incubated with a polyclonal HvCellF6 primary antibody (Wilson et al., 2015) at 1:2000 dilution followed by anti-rabbit horseradish peroxidase (HRP) conjugate (BioRad) at a 1:10 000 dilution. Signal detection was performed with SuperSignal West Pico chemiluminescent substrate (Thermo Fisher Scientific) according to the manufacturer's instructions and using a ChemiDoc MP Imaging System (BioRad).

Tissue preparation for MALDI-MSI measurement

Sectioning of barley grains and matrix application was performed following Peukert et al. (2014). The MS imaging (MSI) measurements were performed using an ultrafleXtreme matrix-assisted laser desorption/ionization (MALDI) time-of-flight (TOF)/TOF device (Bruker Daltonics) following Peukert et al. (2016a).

Tissue fixation, embedding, and microscopy for barley grain

Developing grains were halved using a razor blade and fixed in 0.25% glutaraldehyde, 4% paraformaldehyde, and 4% sucrose in phosphate-buffered saline (PBS), pH 7.2, dehydrated, and embedded in LR White resin following Burton et al. (2011). Embedded tissue was sectioned (1 µm) on an ultramicrotome using a diamond knife and dried onto polylysine-coated microscope slides (Thermo Fisher Scientific). Immunohistochemical analysis of cell wall polysaccharides was performed according to Burton et al. (2011) using primary antibodies including (1,3)-β-glucan (Biosupplies), hetero-(1,4)-β-mannan (Pettolino et al., 2001), BG1 (Biosupplies), LM11 (McCartney et al., 2005), LM19 (PlantProbes), and a recombinant crystalline cellulose CBM3a protein (PlantProbes). For detection of crystalline cellulose, a monoclonal anti-polysaccharidase antibody (Sigma-Aldrich) [1:100 dilution in 1× PBS with 1% (w/v) BSA] was applied after CBM3a (Blake et al., 2006). For detection of arabinoxylan, sections were incubated in α-1,3-arabinofuranosidase (Megazyme International, Deltagen) under moist conditions at 40 °C for 2 h for unmasking of antibody epitopes prior to LM11 antibody
incubation (Wilson et al., 2012). For detection of homogalacturonan, sections were either not treated or treated with 0.1 M sodium carbonate (Na₂CO₃) for 1 h at room temperature for unmasking of antibody epitopes prior to LM19 antibody incubation (Verhertbruggen et al., 2009). Alexa Fluor® 555 goat anti-mouse IgG (H+L) secondary antibody (Thermo Fisher Scientific) was used against BG1, (1,3)-β-glucan, and anti-polyhistidine antibodies, while Alexa Fluor® 555 goat anti-rat IgG (H+L) secondary antibody (Thermo Fisher Scientific) was used against pAsGlo1:HvCslF6 extracts of transgenic and WT grain at 15 DAP, although the predicted size for HvCSLF6 is 105 kDa (Wilson et al., 2015). Levels of HvCSLF6 were greater in transgenic endosperm than in the WT; a weak HvCSLF6 signal was also observed in the 18–6 ‘outer tissues’ sample (Fig. 1A). Out of the four transgenic lines tested, the highest accumulation of HvCSLF6 was observed in line 18–6, which also showed the strongest grain morphological phenotype.

Accumulation of HvCslF6 transcript was measured in WT and transgenic grain tissues at multiple DAP. From 7 to 19 DAP, transcript levels were higher in transgenic ‘outer

Results

*pAsGlo1:HvCslF6 is expressed in the peripheral endosperm and correlates with increased HvCSLF6 protein and MLG abundance*

To determine HvCSLF6 protein abundance, a specific HvCslF6 antibody was used (Fig. 1A). Consistent with

**Fig. 1.** Detection of HvCslF6 protein and YFP fluorescence in developing barley grain. (A) Total HvCslF6 protein levels in the transgenic grains are slightly higher in ‘outer tissues’ and clearly higher in endosperm tissues relatively to the WT at 15 DAP. The arrow indicates HvCslF6 protein at 89–100 kDa. (B–D) Fluorescence from YFP is detected in the peripheral endosperm of barley grain at 20 DAP. Images (B) and (C) are under ×1 magnification with scale bars equivalent to 1000 μm. Image (D) is under ×10 magnification with a scale bar equivalent to 100 μm. Sections for image (C) were prepared in the equatorial part of the grain. “Endosperm” sample includes embryo tissues. Abbreviations: aleurone (al), central endosperm (ce), embryo (em), pericarp (p), peripheral endosperm (pe).
tissues' and endosperm compared with the WT and WT(tc), and they were also slightly higher in 24 DAP transgenic 'outer tissues' compared with the WT. Lowest expression of the HvCslF6 transcript was observed for line 15-3. In the embryo, only the 18-6 transgenic line had higher HvCslF6 transcript levels at 19 DAP relative to the WT (Supplementary Fig. S1A).

To examine tissue specificity of the AsGlo promoter (pAsGlo1), fresh barley grain sections from plants transformed with the pAsGlo1:3xns1YFP transcriptional fusion construct were observed under a fluorescence microscope. Yellow fluorescent protein (YFP) fluorescence was first detected at ~7 DAP (data not shown) and by 20 DAP was seen specifically in the peripheral endosperm of the grain. No signal was detected in aleurone or pericarp tissues (Fig. 1B–D).

To establish whether contamination from the peripheral endosperm tissue had carried over into the manually prepared 'outer tissues' samples, transcript levels of the aleurone-specific gene, lipid transfer protein 2 (HvLtp2) (Kalla et al., 1994) and the HvGBSS1a gene, which is not detectable in barley pericarp (Radchuk et al., 2009), were examined (Supplementary Fig. S1D, E). HvLtp2 transcript expression was detected at similar levels in both endosperm and 'outer tissues' samples, while HvGBSS1a transcript expression was found at a higher level in endosperm preparations. These results indicate that a considerable proportion of peripheral endosperm was present in the manual preparations of 'outer tissues' of all samples. These samples were named 'outer tissues' throughout the manuscript, and they contained pericarp, testa, nucellar epidermis, and some aleurone cells.

Distribution of MLG was examined by immunolabelling of grain sections (Fig. 2A–D). At 7 DAP, BG1 antibody labelling was evenly detected across both WT and 18-6 transgenic grain (data not shown). At 11 and 24 DAP, signals from BG1 antibody labelling were uniformly distributed across the tissue cell walls in WT grain (Fig. 2A, C) whereas for 18-6 transgenic grain, the fluorescence signals were stronger in the peripheral endosperm cell walls (cell walls appeared thicker; Fig. 2B, D), appearing similar to the pAsGlo1:3xns1YFP expression pattern (Fig. 1C). BG1-associated fluorescence also indicated that peripheral and central endosperm cells in transgenic grain were irregularly arranged and the central endosperm cells appeared more 'squashed' (Fig. 2D). Higher BG1 labelling in transgenic grain coincided with increased endosperm MLG content at 24 DAP (Supplementary Fig. S2A, B) and increased MLG content (% w/w) in the mature endosperm in all transgenic lines compared with the WT (Supplementary Table S1A).

**Starch granules are smaller in pAsGlo1:HvCslF6 transgenic grain**

The 'squashed' endosperm cells in 18-6 transgenic grain were packed with starch granules which appeared smaller than A-type (large) but much larger than B-type granules (small) in the WT (Fig. 3A, B). Small starch granules were also found in the peripheral endosperm layer of 18-6 transgenic grain, while the equivalent cells in the WT contained fewer, larger granules...
The size and distribution of starch granules in grain from the WT, WT(tc), and all transgenic lines were further examined (Table 1). Barley grain starch comprises large granules with diameters in the range of 10–40 μm and small granules ranging from 1 μm to 10 μm (Palmer, 1972; Chmelik et al., 2001). Therefore, granules with diameters < 10 μm were grouped as small granules (B-type) while granules with diameters > 10 μm were grouped as large granules (A-type). WT, WT(tc), 15–3, 16–5, and 25–5 had small granules with diameters ranging between 4.3 μm and 4.9 μm, whereas granules from 18–6 had diameters > 5 μm. Line 18–6 had the smallest A-type granules, with average diameters of ~15 μm, whereas those of WT, WT(tc), and other transgenic lines ranged between 20 μm and 26 μm. In transgenic grain, the overall size distribution was shifted to a higher proportion of A-type granules while the median granule size in this group was reduced compared with the WT(tc) and WT grain. Lowest effects on starch granules size and distribution were observed for line 15–3 (Table 1).

### Table 1. Analysis of starch granules in mature barley grain

| Average diameter of starch granules | WT | WT(tc) | F6-15-3 | F6-16-5 | F6-18-6 | F6-25-5 |
|------------------------------------|----|--------|---------|---------|---------|---------|
| A-type large granules (>10 μm)     | 22.99±0.71 | 25.77±0.65* | 22.34±0.99 | 20.64±0.68* | 15.24±0.41* | 20.45±0.25* |
| B-type small granules (<10 μm)     | 4.60±0.12 | 4.93±0.02* | 4.51±0.12 | 4.29±0.21* | 5.49±0.12* | 4.39±0.05* |
| Distribution given as % of granules falling into the respective bins | 7.38±1.44 | 5.87±1.81 | 7.39±1.03 | 10.40±3.62 | 7.27±3.20 | 8.16±1.20 |
| 1–2.5 μm                           | 7.38±0.22 | 1.50±0.20 | 1.80±0.58 | 3.89±0.93 | 3.89±0.93 | 4.43±0.59 |
| 2.5–5 μm                           | 0.61±0.22 | 0.34±0.06 | 1.01±0.60 | 4.19±0.89 | 11.90±1.65 | 4.51±0.42 |
| 5–7.5 μm                           | 0.71±0.35 | 0.33±0.10 | 1.14±0.70 | 4.50±1.59 | 8.11±0.74 | 5.08±0.87 |
| 7.5–10 μm                          | 0.96±0.49 | 0.45±0.16 | 1.32±0.82 | 5.01±1.14 | 5.38±1.54 | 6.17±1.18 |
| 10–12.5 μm                         | 1.33±0.64 | 0.55±0.19 | 1.68±0.98 | 5.51±1.19 | 3.03±1.04 | 7.10±1.25 |
| 12.5–15 μm                         | 1.47±0.71 | 0.75±0.43 | 1.75±0.98 | 5.02±1.50 | 1.57±0.35 | 5.94±1.32 |
| 15–17.5 μm                         | 1.43±0.55 | 1.00±0.44 | 1.63±0.64 | 4.01±0.79 | 0.70±0.34 | 4.35±0.14 |
| 17.5–20 μm                         | 1.16±0.39 | 1.08±0.50 | 1.25±0.53 | 2.38±0.57 | 0.45±0.14 | 2.95±1.01 |
| 20–22.5 μm                         | 0.72±0.24 | 1.10±0.53 | 0.76±0.31 | 1.64±0.39 | 0.17±0.11 | 1.46±0.38 |
| 22.5–25 μm                         | 0.42±0.16 | 0.85±0.38 | 0.48±0.19 | 0.66±0.27 | 0.07±0.06 | 0.68±0.23 |
| 25–30 μm                           | 0.41±0.10 | 0.57±0.36 | 0.21±0.11 | 0.25±0.15 | 0.02±0.02 | 0.26±0.14 |
| 30–32.5 μm                         | 0.08±0.04 | 0.40±0.37 | 0.09±0.04 | 0.10±0.13 | 0.002±0.007 | 0.08±0.07 |
| 40–42.5 μm                         | 0.02±0.02 | 0.19±0.16 | 0.03±0.02 | 0.07±0.11 | 0.003±0.03 | 0.03±0.03 |
| 42.5–45 μm                         | 0.01±0.01 | 0.11±0.08 | 0.02±0.02 | 0.01±0.02 | 0.01±0.02 | 0.01±0.02 |
| 45–47.5 μm                         | 0.00±0.00 | 0.04±0.03 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 |
| >50 μm                             | 0       | 0.02±0.02 | 0.00±0.00 | 0.01±0.01 | 0       | 0       |
| Total % large                      | 9.99   | 8.83    | 12.57   | 37.31   | 45.08   | 43.06   |

The number of evaluated granules per sample varied between 1588 and 21 193; three biological replicates with three technical replicates each were analysed per line. The average diameter of starch granules, grouped into large (A-type, >10 μm) and small (B-type, <10 μm) granules, ± SD is shown in the upper part of the table. An asterisk indicates a significant difference from the WT by t-test with P-values of <0.05. The distribution based on the diameter of starch granules is given in the lower part of the table as % ±SD of granules falling into the respective size categories. A grey scale (dark grey, highest value; white, lowest value) has been applied to highlight the most abundant size proportion within the groups of A- and B-type granules, and the overall percentage of granules per group is given for each line.
At 10 DAP, the ETCs in transgenic grain were ruptured (Fig. 4C, D), becoming increasingly pronounced during subsequent development, whereas the ETCs remained intact in the WT grain (Fig. 4E, F; Supplementary Fig. S3C, D). In WT grain, cell walls of the ETCs were denser and thicker (Fig. 4G) compared with those of transgenic grain which were thinner and surrounded by much less regularly shaped cells (Fig. 4H).

**Altered cell wall composition in the ETCs and endosperm of pAsGlo1:HvCslF6 transgenic grain**

To determine whether ETC rupture might be associated with changes in cell wall composition, sections of WT and 18-6 transgenic grain were examined. From 9 to 11 DAP, the labelling patterns for the mannan antibody were similar between WT and transgenic grain, although the intensity of mannan labelling was higher in the WT (Fig. 4I, J). Labelling of MLG (Fig. 2A, B), callose (Supplementary Fig. S4A, B), crystalline cellulose (Supplementary Fig. S4C, D), and arabinoxylan (Supplementary Fig. S5A, B) was not detected in the ETC walls of either WT or transgenic grain. Arabinoyxlan labelling was greater in the nucellar epidermis of transgenic grain when compared with the WT (Supplementary Fig. S5A, B). Labelling of pectin was detected in the cell walls of the NP, and notably only in the walls of ETCs adjacent to the cavity in both transgenic and WT grain (Supplementary Fig. S6A–D).

Clear differences were observed in the abundance of arabinoxylan, crystalline cellulose, and callose in the endosperm walls of WT and 18-6 grain (Supplementary Fig. S5A–H) where transgenic grain showed stronger labelling for arabinoyxlan and callose relative to the WT (Supplementary Fig. S5A–D). More intense labelling for callose and crystalline cellulose was detected in the peripheral endosperm of transgenic grains relative to the WT, similar to MLG (Fig. 2A–D; Supplementary Fig. S5C–F). Labelling of crystalline cellulose was completely absent in the nucellar epidermis and adjacent cell rows of transgenic grain (Supplementary Fig. S5E, F), as noted for ETCs (Supplementary Fig. S4C, D). The labelling intensity of mannan (Supplementary Fig. S5G, H) was similar between WT and transgenic endosperm, as was that of LM19, which was detected in maternal grain tissues following pre-treatment with 0.1 M sodium carbonate (Supplementary Fig. S6E–H).

**Modified aleurone cell shape in pAsGlo1:HvCslF6 transgenic grain**

At 9 DAP, peripheral endosperm cells in 18-6 and WT grain appeared similarly developed (Supplementary Fig. S7A, B), while at 12–14 DAP, aleurone cells in 18-6 appeared larger relative to the WT (Supplementary Fig. S7C–F). By 24 DAP, they were notably elongated in 18-6 while the equivalent WT cells remained cube shaped (Supplementary Fig. S7G, H). From 14 to 24 DAP, increasing thickness of 18-6 aleurone and peripheral endosperm cell walls was observed relative to the WT (Supplementary Fig. S7E–H) coinciding with increased MLG, arabinoyxlan, callose, and cellulose deposition in these cell walls (Fig. 2C, D; Supplementary Fig. S5A–F).

Measuring aleurone cell shape revealed that at 24 DAP, the average length in 18-6 was $44.8\pm0.2 \mu m$ (SE) and the width was $16.1\pm0.3 \mu m$ (SE). For the WT, the average length was $18.7\pm0.5 \mu m$ (SE) with a width of $17.8\pm0.2 \mu m$ (SE), confirming that WT aleurone cells were cuboid and transgenic.
cells were over twice as long as, but similar in width to, those of the WT. Transgenic aleurone cells remained relatively elongated inwardly compared with the WT at each stage through to grain maturity (Fig. 3D), and the aleurone layer was thicker at maturity in all transgenic lines relative to the WT (Supplementary Fig. S8).

pAsGlo1:HvCslF6 transgenic barley grain accumulated more (1,3;1,4)-β-glucan, sucrose, and total fructan, and less starch in the endosperm compared with the WT

Storage carbohydrate deposition was examined in WT, WT(tc), and transgenic lines from 7 DAP until maturity (Fig. 5A–F). Differences were observed in the MLG content of the WT(tc) lines relative to the WT, whereas the levels of starch, sucrose, and total fructan were similar (Fig. 5A, B; Supplementary Table S1A). Likewise, with the transgenic lines, the greatest variations were observed in 16–8 and the smallest in 15–3, which had similar levels of MLG to WT(tc) per grain but significantly lower levels of starch and WT(tc) grain accumulated starch and MLG throughout grain development (Fig. 5A, B), whilst starch and MLG declined slightly after 24 DAP in transgenic grain (Fig. 5C–F). WT and WT(tc) grain reached maximum total fructan levels at 11 DAP at ~1.2 mg, while transgenic lines ranged between 1.2 mg and 1.5 mg from 11 to 24 DAP (Fig. 5C–F). Sucrose levels in WT and WT(tc) grain were constant from 11 DAP to maturity at 0.5 mg, and increased in transgenic grain from 11 to 19 DAP, from 0.7 mg to 1 mg (Fig. 5C–F). MLG levels were increased in transgenic developing grain from 11 DAP onwards relative to the WT. At maturity on a per grain basis, transgenic grain (except line 16–5 with similar and line 18–6 with lower MLG levels) contained more MLG but less starch than WT grain (Fig. 5; Supplementary Table S1A). While glucose and fructose levels were lower in all transgenic grain samples, sucrose and total fructan levels were comparable with those of the WT and WT(tc) (Supplementary Table S1A). On a weight per weight basis, all transgenic grain contained more MLG, sucrose, and total fructan (except line 15–3 with similar total fructan levels) but less starch than the WT in developing and mature endosperm (Supplementary Figs S2A, C, S9A, C; Supplementary Table S1A). Protein levels were reduced in grain of all transgenic lines on a per grain basis, which is mainly attributed to the reduced endosperm weight relative to WT(tc) grain (Supplementary Table S1). The fraction of other grain constituents, including lipids, cellulose, lignin, and other secondary metabolites (e.g. tocopherol and phytosterols), minerals, and vitamins was higher in mature ‘outer tissues’ and endosperm of transgenic grain (Supplementary Table S1A). Cellulose is likely to be the major component of the grain cell wall, as shown in Supplementary Fig. S5E and F.

Sucrose and fructans accumulate in the developing pAsGlo1:HvCslF6 grain

Dramatic morphological changes in transgenic ETCs coincided with the early phase of storage product formation (9–14 DAP) (Fig. 4A–F; Supplementary Fig. S3A–D), and were followed by pronounced differences in endosperm storage product accumulation from 15 DAP onwards (Fig. 5A–F; Supplementary Figs S2A–D, S9A–D). MALDI-MSI was used to visualize the spatio-temporal distribution of oligosaccharides in WT and 16–8 transgenic grain at both 15 and 24 DAP. Sections were prepared from basal grain parts to also observe the embryo region (Fig. 6A–H). At 15 DAP, accumulation of disaccharides [degree of polymerization 2 (DP2)] in transgenic and WT cavity regions was similar (Fig. 6C), while signals for tri- and tetrasaccharides (DP3 and DP4) were higher in transgenic endosperm and between the endosperm and embryo (Fig. 6E, G). At 24 DAP, DP2 levels were comparable in the cavity and embryo of both transgenic and WT grain (Fig. 6D), whilst DP3 and DP4 signals remained higher in the transgenic grain, mainly adjacent to the cavity and endosperm (Fig. 6F, H).

Quantitative measurement of soluble sugars in cavity fluids revealed that glucose, fructose, sucrose, and total fructan levels, as a proportion of the total sugars from one whole grain, were similar between WT and transgenic grain at 11 DAP (Table 2). At 15 DAP, glucose, sucrose, and total fructan levels increased significantly in the transgenic cavity compared with the WT (Table 2), with transgenic sucrose and total fructan levels higher at 24 DAP (Table 2). At 15 DAP, twice as much volume of cavity fluid was obtained from transgenic lines compared with WT grain (Supplementary Fig. S11).

Carbohydrate metabolic pathways are altered in pAsGlo1:HvCslF6 grain

Transcript levels of genes encoding key enzymes associated with carbohydrate synthesis and encoding proteins with carbohydrate transport roles were measured in ‘outer tissues’, endosperm, and embryo across development. The genes were cell wall-related [HvCslF6, HvCslH1, endo-β-(1,3;1,4)-glucanase E1 (Endo E1), cellulose synthase 2 (CesA2), and cellulose synthase-like F9 (CslF9)], starch-related [GBSS1a, starch synthase 2 (SS 2), SBE2a, SBE2b, Iso1, LD, and sucrose synthase 1 (SuSy)], sucrose metabolism-related [cell wall invertase 1 (CWINV1), vacuolar invertase (VIN), sucrose phosphate synthase (SPS), sucrose phosphate phosphatase (SPP), sucrose transporter 1 (SUT1), and sucrose transporter 2 (SUT2)], and fructan biosynthetic genes (1-SST, 1-FFT, and 6-SFT). HORVU6Hr1G011260 (INV*) and HORVU2Hr1G109120 (INV**) were selected based on their putative role in hydrolysing glycosidic bonds between two carbohydrate molecules, including fructans (Fig. 7; Supplementary Table S2).

Consistent with increased expression from the pAsGlo1:HvCslF6 construct, HvCslF6 transcript was up-regulated in transgenic endosperm at 7 and 11 DAP, declining until 24 DAP, whilst HvCslH1 was up-regulated in transgenic endosperm from 15 to 24 DAP (Fig. 7). In embryo tissues, HvCslF6 was up-regulated at 19 DAP Endo E1, encoding a protein that hydrolyses MLG, was down-regulated in transgenic endosperm and embryo across development (Fig. 7). HvCslF9, encoding another MLG synthase with the highest expression at ~8 DAP in developing grain (Burton et al.,...
Fig. 5. The carbohydrate content: MLG, starch, sucrose, and total fructan during barley grain development on a per grain basis (mg). (A) WT, (B) WT(tc), and pAsGlo1::HvCsFLB transgenic lines (C) 15-3, (D) 16-5, (E) 18-6, and (F) 25-5. For measurements of storage carbohydrates (MLG and starch) from developing and mature grains, ‘outer tissues’ and endosperm were analysed as a single sample. The embryo, containing low amounts of storage carbohydrates, was removed and used for analysis of soluble sugars. For soluble sugar measurements, developing and mature grains were separated into ‘outer tissues’, endosperm, and embryo. The total sum of values is shown. Additional carbohydrate content and statistics for mature grain are presented in Supplementary Table S1 for ‘outer tissues’ plus endosperm as well as for embryo tissues and in Supplementary Figs S2, S9, and S10 for developing grain tissues.
was down-regulated in transgenic endosperm from 7 to 15 DAP relative to the WT (Supplementary Fig. S1B) while *HvCesA2*, encoding a cellulose synthase (Burton et al., 2004; Desprez et al., 2007), showed similar transcript levels in both WT and transgenic endosperm from 7 to 15 DAP and was down-regulated from 19 to 24 DAP (Supplementary Fig. S1C).

Starch metabolic gene transcripts, *SS2, SBE2a, SBE2b, GBSS1a, Iso1, and LD*, were down-regulated in transgenic endosperm across development, with the lowest levels at 24 DAP. In contrast, *SBE1* was highly up-regulated compared with the WT in all transgenic tissues during development (Fig. 7). Sucrose is hydrolysed by cell wall invertase (*CWINV*) and vacuolar invertase (*VIN*). *CWINV1* was down-regulated in transgenic endosperm and embryo during late grain filling from 19 to 24 DAP. In the fructan metabolic pathway, *6-SFT* was up-regulated in the transgenic endosperm from 11 to 19 DAP and in the embryo slightly at 19 DAP, while *6-SST*, *1-SST*, and *1-FFT* were all increased during late grain filling from 19 to 24 DAP relative to the WT (Fig. 7). The *HORVU2Hr1G109120* gene was up-regulated concurrently with the *6-SFT* gene in transgenic endosperm, while *HORVU6Hr1G011260* transcript levels changed oppositely to *6-SFT* (Fig. 7).

The sucrose transporter gene, *SUT1*, was up-regulated in transgenic endosperm at 19 DAP and down-regulated in transgenic embryo at 15 DAP, while *SUT1* and *SUT2* were both slightly up-regulated in transgenic 'outer tissues' at 24 DAP relative to the WT. *SuSy1, SPS*, and *SPP*, all involved in sucrose metabolism, showed only small transcript changes compared with the WT (Fig. 7). During the storage phase overall (15–24 DAP), fructan biosynthetic and sucrose transporter genes were up-regulated, whilst invertase and starch metabolic genes were down-regulated in the transgenic endosperm, coinciding with increased sucrose and fructans and decreased starch contents, as shown in the sugar quantitative data (Supplementary Figs S2C, D, S9A, C, D).

**Discussion**

**Specificity of pAsGlo1 promoter activity in barley grain and HvCslF6 regulation**

Increased endosperm MLG content in pAsGlo1::HvCslF6 lines is consistent with the early activity of the pAsGlo1 promoter shortly after the completion of endosperm cellularization.
specifically in the peripheral endosperm and matching the up-regulation of \(HvCslF6\) transcripts at 7 DAP (Fig 1A–D; Supplementary Fig. S1A). The \(pAsGlo1\)-driven YFP was not detected in transgenic 'outer tissues' or embryo, so higher \(HvCslF6\) transcript levels here may be due to contamination from the peripheral endosperm layer. Similar contamination arising from hand dissection of grain tissues has been reported for endosperm transcriptome data sets (Schon and Nodine, 2017). Based on these observations, we assume that \(HvCslF6\) transgene overexpression occurs exclusively in the endosperm, and not in the 'outer tissues' or embryo.

Previous studies have indicated that regulation of MLG synthesis, and \(HvCslF6\) transcript levels can change in response to varying environmental conditions (Dickin et al., 2011; and reviewed in Houston et al., 2016). Consistent with these previous observations, here we observed that wild type lines that had been through tissue culture [WT(tc)] were similar to the WT in relation to their grain starch, sucrose, and total fructan, but they differed in the MLG content, indicating that there may be feedback from the environmental cues that can influence the regulation of \(HvCslF6\) in grain. Differences in \(HvCslF6\) expression (Supplementary Fig. S1A) are also likely to be the reason for the variation between transgenic lines in their grain MLG, starch, sucrose, and total fructan levels (Fig. 5; Supplementary Table S1).

### Table 2. Percentage of soluble sugar content (glucose, fructose, sucrose, and total fructan) in the endosperm cavity per total corresponding sugar from a single grain at 11, 15, and 24 DAP on a w/w basis

| DAP | Glucose (%w/w) ±SD | Fructose (%w/w) ±SD | Sucrose (%w/w) ±SD | Total fructan (%w/w) ±SD |
|-----|--------------------|---------------------|--------------------|------------------------|
| 11  | 0.33±0.01          | 0.17±0.03           | 0.26±0.02          | 0.28±0.03              |
| 15  | 0.23±0.01          | 0.35±0.34           | 0.48±0.27          | 0.50±0.22              |
| 19  | 0.40±0.32          | 0.26±0.27           | 0.38±0.26          | 0.40±0.26              |

An asterisk indicates a significant difference from the WT by t-test with \(P\)-values of <0.05.

### Altered aleurone development in \(pAsGlo1:HvCslF6\) transgenic barley

The aleurone cells in 18–6 transgenic grain appeared permanently elongated inwardly by 24 DAP (Fig. 3C, D; Supplementary Fig. S7A–H). Differentiation of aleurone cells is initiated at ~8 DAP (Bosnes et al., 1992) driven by signals released from the peripheral starchy endosperm (Becraft and Asuncion-Crabb, 2000; Gruis et al., 2006). A number of genes are involved in regulating aleurone cell fate and tissue differentiation in cereal grains, including \(defective kernel1\) (\(Dek1\)), \(crinkly4\) (\(Cr4\)), and \(supernumerary aleurone1\) (\(Sal1\)) (Lid et al., 2002; Olsen et al., 2008). Aleurone development is also associated with hormonal regulation (Bethke et al., 1999; Gómez-Cadenas et al., 2001; Geisler-Lee and Gallie, 2005) whilst aleurone cell number has been linked to endosperm and embryo development as well as to differential hydrolytic enzyme activity (Aubert et al., 2018). For example, 5\textsuperscript{d} maize mutants have more aleurone cells, a shrunken endosperm with reduced starch granule density, and a smaller embryo (Lid et al., 2004); \(defective seed5\) (\(des5\)) barley mutants have fewer aleurone cells, a shrunken endosperm, and altered starch granule types, and embryos prematurely abort (Olsen et al., 2008).

Peripheral endosperm cells containing more \(HvCslF6\) transcript and HvCslF6 protein appear to be structurally different from the WT from 9 DAP onwards (Figs 2A–D, 3A–D). The thicker cell walls in transgenic aleurone (Fig. 4E–H) relative to the WT probably result from an extended period of MLG deposition in the peripheral endosperm cells, as also shown for \(Brachypodium distachyon\) (Trafford et al., 2013). Due to the specific grain size, cell expansion is only possible towards the middle of the grain, resulting in inwardly elongated aleurone cells and 'squeezed' adjacent starchy endosperm.
**Morphology and cell wall composition changes in pAsGlo1: HvCslF6 ETCs**

A larger cavity and unevenly formed ETC layer were observed in transgenic grain relative to the WT from 9 DAP onwards, and the ETCs began to rupture at 10 DAP (Fig. 4C, D; Supplementary Fig. S3A, B). At this stage, the ETC walls in transgenic grain showed less intense mannan labelling than the WT (Fig. 4L, J). In the ETC walls, no MLG, arabinoxylan, callose, or crystalline cellulose was detected (Supplementary Fig. S5A–D), while the labelling of pectin probably relates to the remnants from the nucellus (Supplementary Fig. S6–H) (Greenwood et al., 2005).

These results do not match the transcriptome analysis by Thiel et al. (2012), who demonstrated up-regulation of genes related to callose and cellulose metabolism at the same time point (10 DAP). The failure of antibodies to detect cell wall polysaccharides other than mannan could be due to epitope masking, making them unavailable for binding, as has been reported by Xue et al. (2013). Closer examination of the cell wall polysaccharides in ETCs may be achieved using immunogold labelling and TEM, with appropriate unmasking techniques. Alternatively, laser microdissection of the ETCs and surrounding endosperm would allow examination of gene expression and link it directly to cell wall composition.

**Altered ETC morphology may trigger abnormal accumulation of sucrose and fructans in the grain cavity and endosperm**

Correct formation of ETCs is important to ensure nutrient transfer from the cavity to the endosperm for development and storage of carbohydrates. A study by Felker et al. (1985) showed that some barley mutants with abnormal endosperm had missing ETCs, such as seg2, seg4, seg5, and seg8, and starch filling in the endosperm was also perturbed. Here, in HvCslF6 transgenic grain during the early storage phase, a significant increase in glucose, sucrose, and fructan content in the cavity and endosperm compared with the WT was observed, whilst starch levels were reduced (Figs 5C–F, 6C–H; Table 2; Supplementary Figs S2C, S9A–D, S11A–D). We hypothesize that the high sucrose and fructan pools in the cavity at 15 DAP may be due to earlier perturbations in ETC differentiation, which may have subsequently perturbed the active flow of sucrose towards the endosperm. A link between changed carbohydrate partitioning and altered development of the transfer region has been suggested previously (Vijan and Smeekens, 1999; Weichert et al., 2010; Ruan, 2014). However, a detailed histological examination of ETC development in pAsGlo1:HvCslF6 grain is required to confirm changes in ETC identity and function. In contrast, the lys5 mutant Risø13, harbouring a mutation in the plastidial ADP-glucose
transporter resulting in reduced starch biosynthesis, did not show such morphological changes or spatial alterations of the sucrose allocation, but decreased velocity of the sucrose stream through the cavity, as compared with the WT (Melkus et al., 2011). Thus, the wrinkled phenotype of mature Riso13 grains is possibly associated with decreased starch deposition in the endosperm and the strongly altered starch granule morphology (Patron et al., 2004).

| Transition phase (6 to 8 DAP) | Storage phase (9 to 23 DAP) | Mature phase |
|-------------------------------|-------------------------------|--------------|
| **Induced HvCslF6 gene transcription (e & pe, 7 to 24 DAP)** | **Increased HvCslF6 protein and MLG biosynthesis** (e & pe, from 10 DAP on) | **Significantly increased MLG level (e)** |
| **Induced sucrose metabolism and transporter gene transcripts** (p and e, 7 to 24 DAP) | **Increased levels of glucose (15 DAP) and sucrose (15 to 24 DAP) in c and e** | |
| **Induced fructan metabolism gene transcripts (p and e, 7 to 24 DAP)** | **Increased levels of total fructans (c and e, 15 to 24 DAP) afterwards declining** | |
| **Reduced starch biosynthesis gene transcripts, except SBE1** (e, 7 to 24 DAP) | **Reduced starch levels (e, from 15 DAP)** | **Densely packed, cell walls not apparent, medium size granules (pe)** |
| | | |
| Poorly formed etc (8 DAP) | Thinner cell walls and reduced mannan staining of etc (10 DAP) | |
| | Rupture of etc borders at several regions (10 to 14 DAP), red arrows | **Significant cavity expansion (c)** |
| | | Shrunken endosperm phenotype after desiccation |
| | | Elongated thicker aleurone cells (al) |

Fig. 8. Schematic diagram of grain development of the pAsGlo1:HvCslF6 transgenic line (A–C) relative to the wild type. (A) In transgenic endosperm, the early up-regulation of HvCslF6 gene expression relative to the WT from 7 DAP results in an increased amount of (1,3;1,4)-β-glucan (MLG) during the grain storage phase, which is followed by a decreased amount of starch and increased levels of glucose, sucrose, and total fructans. Early changes in carbohydrate biosynthesis allocation appear to influence endosperm transfer cell (ETC) differentiation and subsequently perturb sucrose uptake in the endosperm. (B) Consequently, high amounts of sucrose and fructans are accumulated in the grain cavity, causing the cavity to aberrantly expand and subsequent rupture (as indicated by the dotted line). Thinner cell walls in the non-uniformly developed ETCs of transgenic grain and variations in mannan labelling may contribute to ETC rupture in transgenic grain. (C) The transgenic grain has an elongated aleurone layer compared with the WT grain which persisted to maturity. As the grain matures, fluid in the transgenic cavity dries out, causing the grain to become shrunken. High levels of MLG and low levels of starch persist in the mature endosperm. Abbreviations: aleurone (al), cavity (c), endosperm (e), endosperm transfer cells (etc), nucellar projection (np), pericarp (p), peripheral endosperm (pe). Red arrows indicate probable osmotic pressure exerted from the cavity towards the endosperm. Grey boxes indicate findings at the genetic and metabolic level, while blue boxes indicate findings at the morphological level. Dark grey boxes highlight the mature phenotype. Arrangement of boxes roughly reflects observation time during the course of grain development.
Fructans in the endosperm cavity are involved in detoxification of hydroxyl radicals (Peukert et al., 2014; Matros et al., 2015) and maintaining the sucrose gradient between the vascular bundle and sink tissues (Pollock and Cairns, 1991; Peukert et al., 2016b). In 18-6 transgenic grain, ETCs were irregularly formed and the sucrose uptake pathway was likely to have been compromised. At the same time, the increased demand for glucose during MLG synthesis in the endosperm would be likely to have stimulated sucrose import. Fructan synthesis can be induced by sucrose (Vijn and Smeekens, 1999; Ruan, 2014), towards keeping the levels of free fructose temporarily low. In the transgenic endosperm, the fructosyltransferase gene, 6-SFT, is up-regulated, encoding an enzyme catalysing the formation of branched and levan-type fructans containing β(2,6) linkages (Vijn and Smeekens, 1999). Down-regulation of CWINV1 and VIN in the transgenic endosperm may also contribute to the increased amounts of sucrose and fructan, and small amounts of free glucose.

**Starch metabolism is perturbed in pAsGlo1::HvCslF6 endosperm**

*pAsGlo1::HvCslF6* grain showed up-regulation of the glucan synthase HvCslH1 and down-regulation of the endo-glucanase E1 in the developing endosperm (Fig. 7), probably resulting from increased abundance of metabolic precursors and constitutively induced MLG synthesis, respectively. High MLG content in barley is likely to be lowered by lower starch levels as previously reported (Munck et al., 2004; Seefeldt et al., 2009; Burton et al., 2011) and, as seen here, linked to the down-regulation of some starch biosynthetic genes (Fig. 7). This coincides with results from *B. distachyon* where MLG accumulated as the main storage carbohydrate in cell walls during grain development at the expense of starch in mature grain (Guillon et al., 2011). The low capacity for starch synthesis in *Brachypodium* is reflected by reduced transcription of SSI1 and SBE1 as well as reduced activity of starch synthase and ADP-glucose pyrophosphorylase (Trafford et al., 2013). In our study, SBE1 was up-regulated in transgenic endosperm. Previously, SBE1 was found to be involved in producing long-chain amylopectin in rice (Satoh et al., 2003) and in maize (reviewed in Tetlow and Emes, 2014). However, alterations in amylopectin chain length have not been investigated in our study and it is unclear whether up-regulation of SBE1 contributes to overall starch granule size differences seen in barley (Fig. 3A, B; Table 1). Recently, RNAi suppression of all three HvSBE isoforms was shown to result in a severe amylose-only phenotype with irregular granules, while hyperphosphorylation caused WT-like structures (Shaik et al., 2016). Further investigations need to include the amylose/amylopectin ratio and starch phosphorylation status in *pAsGlo1::HvCslF6* grain.

**Conclusions**

Increased MLG levels are desirable for human health benefits, but high levels in barley grain from early stages perturb carbohydrate metabolism, leading to impairment in grain development (Fig. 8). Major changes affect the cell wall composition and morphology of the peripheral endosperm regions (aleurone and adjacent endosperm cells), and, notably, of the ETC layer. The increased MLG synthesis in transgenic grain is compromised by reduced starch and increased fructan synthesis. The high demand for maternally derived sucrose together with the disturbed active import to the endosperm via the ETC border probably result in excessive accumulation of sucrose and fructans in the cavity. The shrunken phenotype of the *HvCslF6* transgenic grain is likely to be due to the increased amount of cavity fluids and higher osmotic potential, forcing water uptake and causing wrinkling due to greater water loss during desiccation. Our investigations of the metabolic, transcriptional, and morphological changes associated with increasing MLG early in grain development (this report) or systemically (Burton et al., 2011) have shown the importance of regulation of carbohydrate metabolism homeostasis in controlling grain phenotypes, suggesting that these transgenic approaches may not be an ideal avenue in pursuit of this outcome. Engineering of barley lines with MLG synthesis inducible at the grain-filling stage as well as selection for high MLG lines from available barley mutant collections may overcome these limitations in the near future.

**Supplementary data**

Supplementary data are available at JXB online.

**Fig. S1.** Normalized transcript levels of *HvCslF6* transgene, *HvCslF9*, *HvCesA2*, *HvLtp2*, and *HvGBSS1a* in developing grain tissues.

**Fig. S2.** MLG and starch contents in developing grain.

**Fig. S3.** Morphology of the endosperm cavity in WT and 18–6 transgenic grain at 9 and 14 DAP.

**Fig. S4.** Immunohistochemical analysis of callose and crystalline cellulose in ETC walls at 10 DAP.

**Fig. S5.** Immunohistochemical analysis of arabinoxylan, callose, crystalline cellulose, and mannan in endosperm cell walls at 10 DAP.

**Fig. S6.** Immunohistochemical analysis of pectins with LM19 antibody on barley grain sections at 10 DAP.

**Fig. S7.** Morphology of aleurone cells in developing WT and 18–6 transgenic grain.

**Fig. S8.** Morphology of aleurone cells in WT and 18–6 transgenic grain at grain maturation and during grain development.

**Fig. S9.** Sucrose and total fructan contents in developing grain. **Fig. S10.** Percentage of soluble glucose and fructose contents in developing grain on a w/w and per grain basis (mg).

**Table S1.** Carbohydrate content in mature grain (embryo removed) and embryo. **Table S2.** List of primers used for real-time PCR.

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