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Introduction of glucan synthase into the cytosol in wheat endosperm causes massive maltose accumulation and represses starch synthesis

Tina B. Schreier[1,2], Brendan Fahy[1], Laure C. David[1,3], Hamad Siddiqui[1,4], Roger Castells-Graells[1,5] and Alison M. Smith[1,*]

[1]*John Innes Centre, Norwich Research Park, Norwich NR4 7UH, UK,*
[2]*Department of Plant Sciences, University of Cambridge, Downing St, Cambridge CB2 3EA, UK,*
[3]*ETH Department of Biology, Universitätstrasse 2, Zurich 8092, Switzerland,*
[4]*Germains Seed Technology, Lab 7, Centrum, Norwich Research Park, Norwich NR4 7UG, UK, and*
[5]*Department of Chemistry and Biochemistry, University of California, Los Angeles, CA 90095, USA*

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*For correspondence (email: alison.smith@jic.ac.uk).

SUMMARY

We expressed a bacterial glucan synthase (*Agrobacterium GlgA*) in the cytosol of developing endosperm cells in wheat grains, to discover whether it could generate a glucan from cytosolic ADP-glucose. Transgenic lines had high glucan synthase activity during grain filling, but did not accumulate glucan. Instead, grains accumulated very high concentrations of maltose. They had large volumes during development due to high water content, and very shrivelled grains at maturity. Starch synthesis was severely reduced. We propose that cytosolic glucan synthesized by the glucan synthase was immediately hydrolysed to maltose by cytosolic β-amylase(s). Maltose accumulation resulted in a high osmotic potential in developing grain, drawing in excess water that stretched the seed coat and pericarp. Loss of water during grain maturation then led to shrinkage when the grains matured. Maltose accumulation is likely to account for the reduced starch synthesis in transgenic grains, through signalling and toxic effects. Using bioinformatics, we identify an isoform of β-amylase likely to be responsible for maltose accumulation. Removal of this isoform through identification of TILLING mutants or genome editing, combined with co-expression of heterologous glucan synthase and a glucan branching enzyme, may in future enable elevated yields of carbohydrate through simultaneous accumulation of starch and cytosolic glucan.

Keywords: wheat grain, bacterial glucan synthase, endosperm development, maltose, starch, *Triticum aestivum*.

INTRODUCTION

There is a pressing need to increase yields of the world's major cereal crops to meet the needs of the growing population and to combat the negative effects of climate change. Although manipulation of rates of photosynthesis can contribute to this goal, it is generally agreed that sink limitation (i.e., the capacity of grains to store assimilates transported from the leaves) is an important factor in determining yield. Numerous attempts have been made to improve sink capacity through manipulation of the rate of synthesis of starch, the major component of cereal grains. It is argued that a higher capacity to sequester incoming assimilate as starch will result in more/larger grains and hence improved yield, although there is little evidence for a strong correlation between grain weight and grain starch content (Cakir *et al.*, 2015; Fahy *et al.*, 2018).

Efforts to increase starch synthesis have focused largely on increasing the activity of ADP-glucose pyrophosphorylase (AGPase), the first committed enzyme of starch synthesis and the source of the ADP-glucose (ADPGlc) substrate for starch synthases (Tuncel and Okita, 2013). Unlike the situation in other plant organs in which AGPase is exclusively plastidial, AGPase activity in the endosperm of the grain is largely cytosolic. Its ADPGlc product is transported from the cytosol into the plastid for starch synthesis. Unfortunately, attempts to increase yield by increasing cytosolic AGPase activity (through expression of more

AGPase protein or AGPase with altered regulatory properties) have met with limited success. Higher grain weights in controlled conditions have not been shown to result in significant yield improvement in the field except under some stress conditions, including high temperatures immediately after pollination (Hannah *et al.*, 2017; Kang *et al.*, 2013; Smidansky *et al.*, 2002; Tuncel and Okita, 2013). Here we describe the first steps towards employing an alternative strategy to increase grain sink strength, by introducing enzymes that can convert the cytosolic AGPGlc into a cytosolic glucan. This approach might potentially provide a second major sink for ADPGlc, allowing synthesis of both starch and glucan and thus a higher total capacity for carbohydrate storage.

Support for such an approach comes from a previous study of leaves expressing cytosolic enzymes of glucan synthesis. Transient expression of yeast or red-algal glycogen-synthesizing enzymes in the cytosol of *Nicotiana benthamiana* leaf cells resulted in accumulation of levels of glucan many times higher than the levels of starch in these cells (Eicke *et al.*, 2017). Eicke *et al.* expressed UDPGlc-dependent glycogen synthases that presumably used the endogenous cytosol UDPGlc pool as the substrate for glucan synthesis. It seems possible that stable rather than transient expression of these enzymes will have a negative impact on endogenous pathways that use UDPGlc, including cell wall synthesis. The fact that cereal endosperm has an endogenous ADPGlc pool entirely committed to storage carbohydrate synthesis meant that we could introduce an ADPGlc-dependent glycogen synthase with a low risk of interference with other metabolic pathways. Accordingly, we expressed ADPGlc-dependent *GlgA*-encoded glycogen synthase of *Agrobacterium* in cytosol of developing grain, an enzyme shown previously to be highly active when expressed in Arabidopsis (Crumpton-Taylor *et al.*, 2013; Lu *et al.*, 2018) and reported to initiate glucan synthesis without a requirement for a primer (Ugalde *et al.*, 2003).

RESULTS

Wheat plants cv Cadenza were transformed with a construct containing the gene encoding the glycogen synthase of *Agrobacterium tumefaciens*, *GlgA*, with expression driven by the promoter of a high molecular weight glutenin subunit, 1Dx5 (Lamacchia *et al.*, 2001). We recovered 30 independent transformants that were shown by polymerase chain reaction (PCR) to contain the *GlgA* gene. We also selected as a control a line that was positive for the gene encoding the selectable marker, bar, but not for the *GlgA* gene. In some experiments, the untransformed parental line Cadenza was included as an additional control. From the transgenic plants containing the introduced *GlgA* gene, we chose three lines in which there was a segregation of 1:2:1 in the T1 generation for plants homozygous, heterozygous and null for *GlgA*. Two of these lines had single insertions of the transgene (lines 1 and 3); the third had multiple insertions at a single locus (five insertions, line 2). The three lines are referred to below as GlgA lines 1, 2 and 3.

Using quantitative PCR, we found that the transcript for *GlgA* was detectable in endosperm of developing grain on T1 plants at the late soft dough stage [30 days after anthesis (DAA) in controlled environment room conditions] for all three lines. No *GlgA* transcript was detectable in the bar-only control or in Cadenza (Figure 1a). To discover whether the GlgA lines contained active glycogen synthase, we measured the activity of starch/glucan synthase in the same pools of developing grain used for quantitative PCR analysis (Figure 1b). Activity in control lines was 28 nmol min^{-1} g^{-1} fresh weight, in line with values in the literature for soluble starch synthase activity at similar stages of grain development [e.g. mean values for field-grown genotypes in the range 15–40 nmol min^{-1} g^{-1} fresh weight (Fahy *et al.*, 2018); values for plants in controlled environment rooms approximately 25 nmol min^{-1} g^{-1} fresh weight in two separate studies (Hawker and Jenner, 1993; Borrill *et al.*, 2015)]. Activity in homozygous transgenic lines was between 8 and 40 times higher than in control lines. As expected, activity in pools of grains from heterozygous plants was lower than that from homozygous plants, but higher than that from control lines.

We used the same pool of grains for a preliminary analysis of the effects of elevated glycogen synthase activity on starch synthesis. Starch content was up to 93% lower in homozygous lines expressing glycogen synthase than in control lines. The activity of AGPase, the first committed enzyme of starch synthesis, was strongly reduced in homozygous transgenic grains, by up to 80%, relative to grains of the bar control (Figure 1c,d). Starch content and AGPase activity were also reduced in heterozygous relative to control grains, but the reductions were less severe than for grains from homozygous plants.

At maturity, grains from homozygous GS lines were markedly different from those of the control line and Cadenza. They were severely shrunken, and had low weights and very low starch contents (Figure 1e–g). Whereas starch accounted for about 67% of the weight of control grains, it made up less than 10% of the weight of grains on homozygous T1 plants. The effect of the transgenes on starch content at maturity was equally strong in grains that developed on T3 homozygous plants (see text below). Grain weight and starch content at maturity were also reduced in heterozygous plants, but the reductions were less severe than for homozygous plants (Figure 1e-g). The grains of homozygous GlgA lines germinated more slowly than those of the control line and Cadenza did, but overall germination success was not affected (Figure 1h).

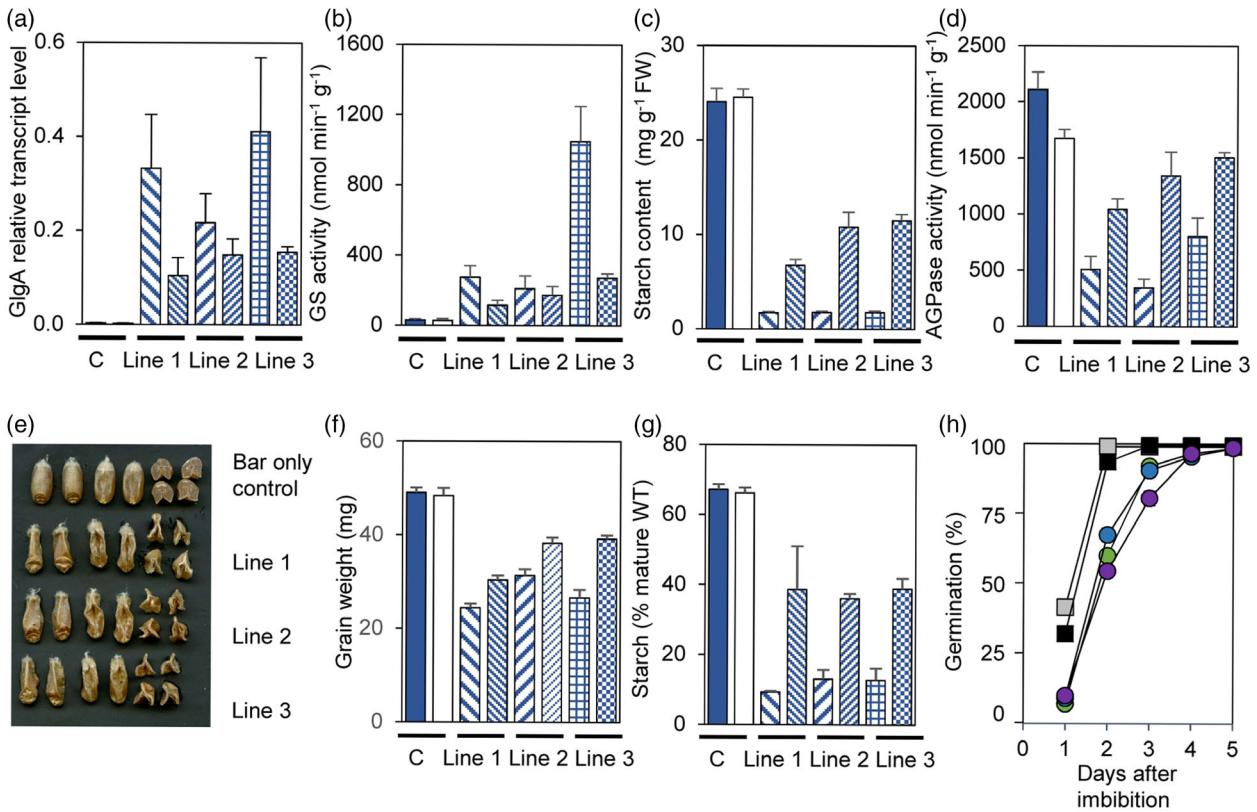

Figure 1. Evaluation of transgenic lines.
T1 plants were grown in a controlled environment room in 16-h days (20°C), 8-h nights.
(a–d) show grains at late soft dough stage and (e–g) show grains at maturity. In (a–d,f,g) large stripes and squares are grains from homozygous lines; small stripes and diamonds are grains from heterozygous lines. Bars marked C are controls; solid blue is the bar-only control and white is the parent line Cadenza. Values for (a–d) are means ± SE from the following numbers of independent samples, each a pool of 10–15 grains from a different plant: (a) 4; (b–d), 5. Enzyme activities are nmol min^{-1} g^{-1} fresh weight (FW). All values for homozygous and heterozygous transgenic lines are statistically significantly different from values for the bar-only control line (Student's *t*-test, $P < 0.05$). In (f) values are means from four pools of grains, each containing 50 grains. Values for transgenic lines are statistically significantly different from those of control lines except at 4 and 5 days after germination. (a) *GlgA* transcript levels, measured with the quantitative polymerase chain reaction.
(b) Glucan synthase activity, measured as [^{14}C]glucose incorporation from ADP[^{14}C]glucose.
(c) Starch content.
(d) AGPase activity, measured as Glc1P production from ADP-glucose plus pyrophosphate.
(e) Appearance of mature grains. Top view (left two), crease view (central two), and cross-sectional view (right).
(f) Average weight of mature grains, measured as thousand grain weight with a MARViN seed analyser.
(g) Starch content of mature grains, as % grain weight.
(h) Germination of GlgA, Cadenza and bar control lines. Values are mean germination percentages ± SE (not visible because they are smaller than symbols) from four independent experiments, in each of which 10 grains were measured at each time point. Germination was defined as the presence of an emerged radicle of at least 5 mm. Genotypes were: Cadenza (grey symbols); bar-only control (black); transgenic lines 1 (green), 2 (blue) and 3 (purple). WT, wild type.

Glycogen synthase activity perturbs dry matter, starch and water content during grain development

To uncover the reasons for the very large reductions in starch accumulation and hence in grain weight in lines with cytosolic glycogen synthase, we examined changes in glycogen synthase activity through grain development and compared these with changes in dry matter, water content and carbohydrates. These analyses were made on grain developing on homozygous T2 plants grown in a glasshouse in summer (between July and September, day lengths 16.5–14 h).

Glycogen synthase activity was assessed on soluble extracts of grains subjected to electrophoresis on native gels containing glycogen, which were then incubated with ADPGlc followed by staining with iodine to reveal bands of glycogen synthase activity. Activity in control and GlgA lines was low at an early stage of grain development, and then it started to rise in GlgA lines. Activity was much higher in GlgA than control lines from 7 to 20 DAA, then declined to a low level as grains approached maturity at 30 DAA (Figure 2a).

Up to about 15 DAA, the dry weights and water contents (percentage of fresh weight) of GlgA grains were not markedly different from those of controls. However, beyond this point, dry matter accumulation was much more rapid in the control than in GlgA grains so that by 30 DAA the dry weight of GlgA grains was only two-thirds that of the

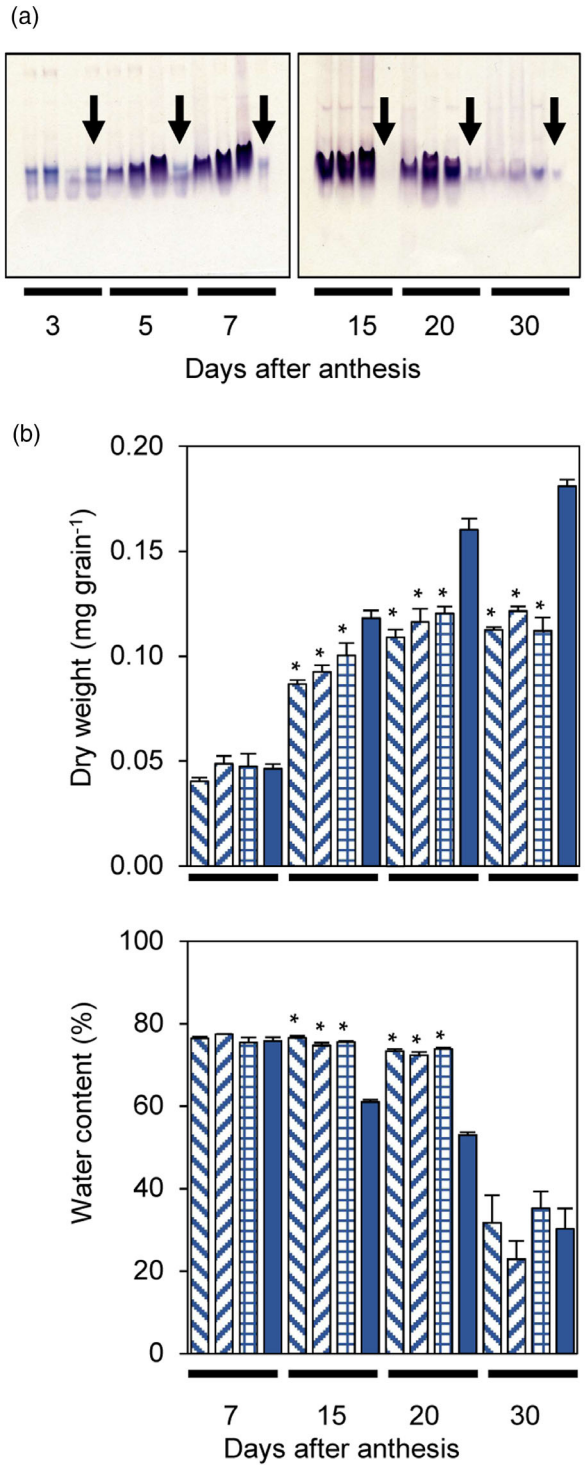

Figure 2. Glucan synthase activity, grain weight and grain water content through development.
(a) Glucan synthase activity visualized on native gels containing glycogen. At the developmental time points indicated, soluble extracts of grains were subjected to electrophoresis on native glycogen-containing gels that were then incubated with ADPGlc and stained with iodine. At each time point, the four lanes show extracts from (left to right) lines 1, 2 and line 3, and a control line (indicated with an arrow). Each lane contained extract equivalent to 4 mg fresh weight.
(b) Water content (upper graph) and dry weight (lower graph) of grains at the developmental time points indicated. At each time point, bars show (left to right) lines 1, 2 and 3, and control (solid blue) values. Values are means ± SE of measurements on five biological replicates, each from a single ear of a different plant. Asterisks indicate values for transgenic lines that are statistically significantly different from the control value at the same time point ($P < 0.05$, Student's *t*-test).

control grains (Figure 2b). The opposite trend was observed for water content: control grains started to lose water between 7 and 15 DAA whereas GlgA grains did not lose water until after 20 DAA (Figure 2b). By 30 DAA, grains of both the control and the GlgA lines had lost a large fraction of their water contents.

Developing GlgA grains have very low starch and exceptionally high sugar contents

The low dry weights and starch contents of mature GlgA grains were attributable to an almost complete cessation of starch accumulation after the early stages of grain filling (Figure 3a). Electron and light microscopy of developing grains confirmed that starch granules were much smaller and less abundant in grains from GlgA than control lines (Figure 4). Granules in grains of GlgA and control lines were of similar dimensions at 6 DAA, but by 15 DAA, granule size was greater in the control than in GlgA grains. At maturity, scanning electron microscopy revealed large numbers of A and B granules in cells of control grains but very small numbers of granules in largely 'empty' cells in grains of GlgA lines.

The high water content of grains of GlgA lines at 15 and 20 DAA were associated with marked swelling of the grains relative to controls in this time interval (Figure 5a). At 20 DAA, grains from GlgA lines had a pocket of liquid beneath the integuments around the crease region, which pushed the vascular strand outwards. In cross-section, the region occupied by the liquid pocket appeared as a large empty space above the nucellar projection (Figure 5b).

These data suggested that GlgA grains might accumulate large amounts of water-soluble metabolites in place of starch, increasing grain osmotic potential and thus drawing in excess water. We measured metabolites on a fresh weight basis. On a dry weight basis, the differences in metabolite levels between transgenic and control lines would be greater at 15 and 20 DAA because of the higher water contents of transgenic lines at these points (Figure 2b). The features of the data described below were seen on both a fresh weight and a dry weight basis.

Sucrose and hexose levels in endosperm tissue were higher in GlgA than control grains at 15 DAA, and substantially higher at 20 and 30 DAA (Figure 3b–d). The higher

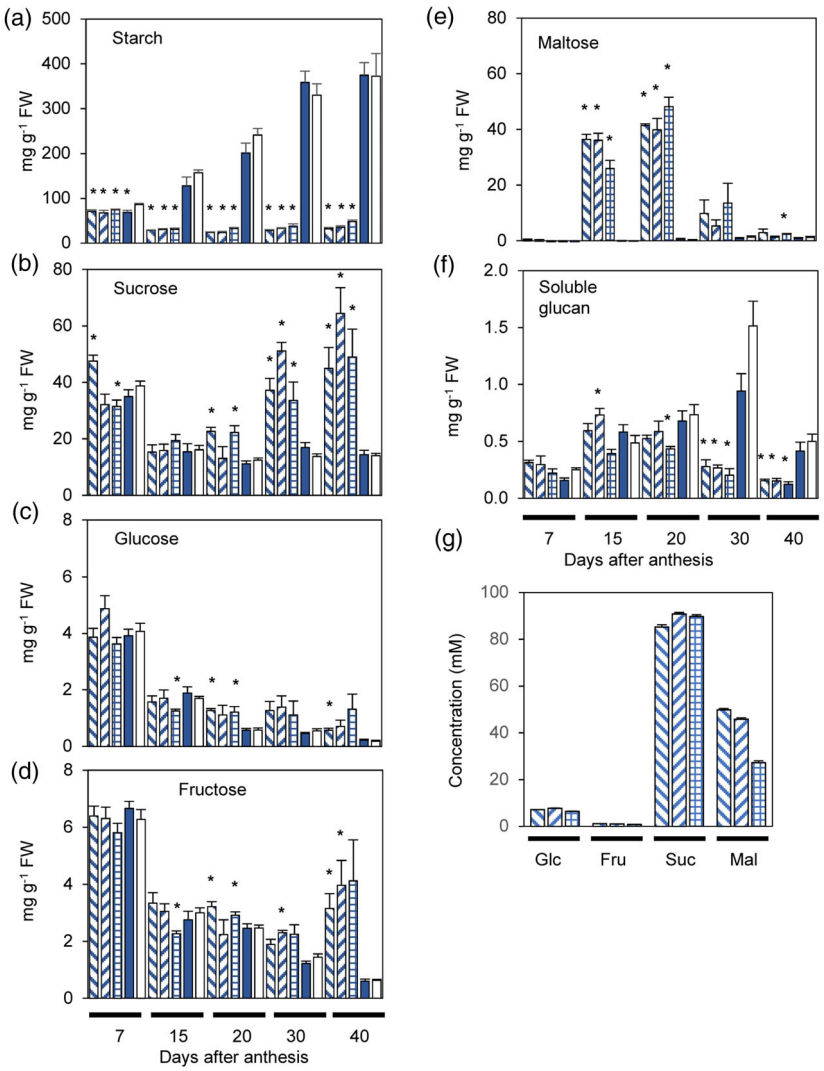

Figure 3. Metabolite levels during grain development.
At each developmental time point, bars show (left to right) lines 1, 2 and 3, bar-only control (solid blue) and Cadenza (white) values. Values are means ± SE of measurements on five biological replicates, each from a single ear of a different plant. Asterisks indicate values for transgenic lines that are statistically significantly different from the Cadenza value at the same time point ($P < 0.05$, Student's *t*-test).
(a) Starch.
(b) Sucrose.
(c) Glucose.
(d) Fructose.
(e) Maltose: below the limit of detection [(0.6 mg g^{-1} fresh weight (FW)] at 7 days after anthesis (DAA) and for control and Cadenza lines at 15 and 20 days DAA.
(f) Soluble glucans, measured on material precipitable with methanol from metabolite extracts.
(g) Concentrations of sugars in the liquid accumulating in the pocket below the nucellar projection in transgenic lines. Liquid from five to seven grains at 20 DAA, taken from three to five plants per line, was pooled and samples were assayed for sugars. Glc, Fru, Suc and Mal are glucose, fructose, sucrose and maltose respectively. Bars represent (left to right) lines 1–3. Values are means of three determinations from a single pool of liquid.

total sugar levels were largely contributed by elevated contents of sucrose. Although there was a general elevation of sucrose and hexoses in GlgA grains, both the extent and timing of this elevation indicated that it could not account for the very substantial swelling and liquid accumulation in GlgA grains. In particular, the swelling of GlgA grains was obvious by 15 DAA, but substantial elevation of sucrose and hexoses was not seen until 20 DAA. We reasoned that GlgA grains might be accumulating a different soluble metabolite as a direct consequence of cytosolic glucan synthase activity. We first tested whether the grains accumulated soluble glucan. Treatment of extracts containing soluble metabolites with methanol-KCl solution (a standard method for glucan precipitation) revealed that levels

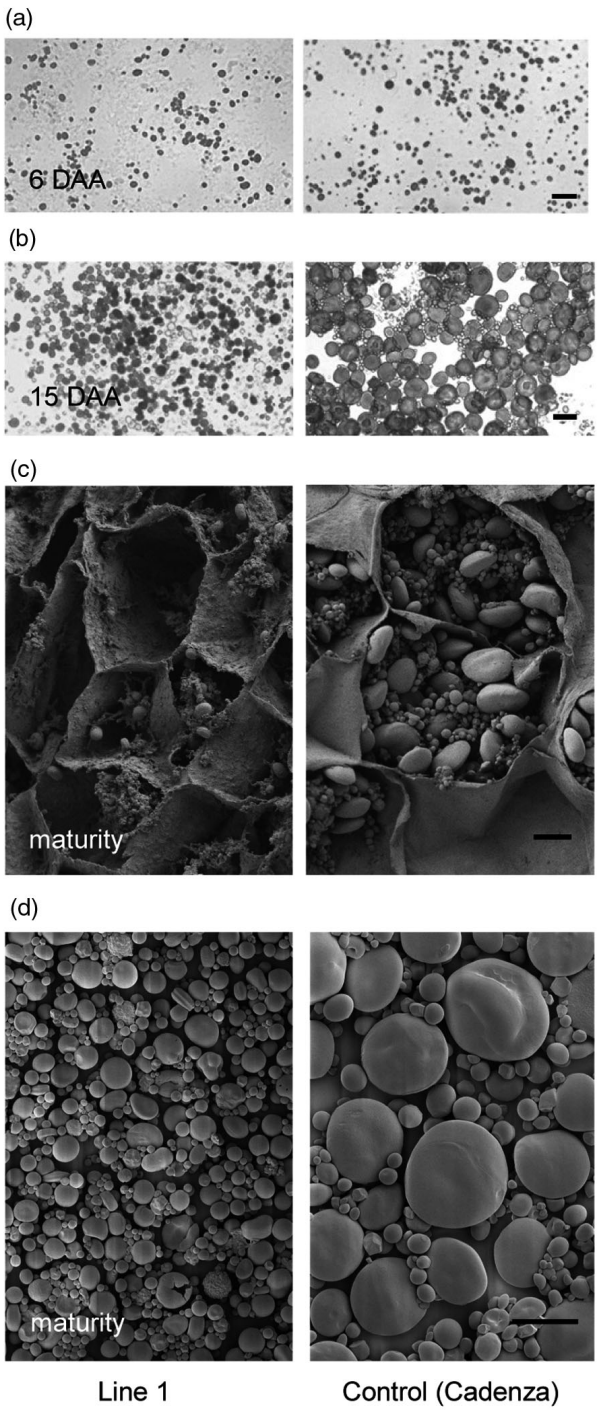

Figure 4. Appearance of starch granules in developing and mature grains.
(a,b) Light micrographs of isolated starch granules at the time points indicated, following staining with dilute iodine solution. DAA, days after anthesis.
(c) Scanning electron micrographs of fracture faces of endosperm in mature grain. Note the 'empty' cells in transgenic line 1 and the starch-filled cells in the parental line Cadenza.
(d) Scanning electron micrographs of starch granules isolated from mature grains. Very similar results were obtained from transgenic lines 2 and 3. Bars represent 20 μm.

of precipitable, soluble glucan were low in grains from both control and GlgA lines (Figure 3f). The higher values in control lines at 30 and 40 DAA are probably due to precipitation of small amounts of starch that remained in the soluble fraction in these high-starch lines. We reasoned that failure to detect a glucan product of cytosolic glycogen synthase might be because the product was subject to hydrolysis by cytosolic β-amylases, resulting in accumulation of maltose in endosperm cells. This hypothesis was confirmed by measurements of maltose using a specific assay that employs heterologously expressed disproportionating enzyme (DPE2) from Arabidopsis (Smirnova *et al.*, 2017) (Figure 3e). Control grains contained very low or undetectable maltose levels throughout development. This was also true in GlgA lines at 7 DAA, but levels of maltose then rose rapidly so that by 15 DAA levels were comparable with or higher than those of sucrose. Levels were even higher, and higher than levels of sucrose – in GlgA lines at 20 DAA. By 30 DAA maltose levels had fallen steeply, and were three- to four-fold lower than levels of sucrose in the GlgA lines at this late developmental stage.

Finally, we investigated the composition of the liquid in the pocket that appeared above the nucellar projection in GlgA lines at 15 and 20 DAA. A fine syringe was used to extract this liquid from five to seven grains at 20 DAA. The collections were pooled for each line, and sampled for sugar measurements. The liquid contained high concentrations of sucrose (85–90 mM) and maltose (27–50 mM), and much lower concentrations of glucose and fructose (7 and 1 mM respectively) (Figure 3).

Developing grains contain transcripts for a cytosolic β-amylase

Given that maltose is the major product of degradation of linear glucans by the exoamylase β-amylase, we examined whether developing wheat grains contain a β-amylase that might be responsible for maltose production in our transgenic lines. The rice β-amylase *Os*BAM1 (LOC_Os07g35940 or Os07g0543300) and the Arabidopsis β-amylase *At*BAM5 are reported as cytosolic (Hirano *et al.*, 2011; Laby *et al.*, 2001). Both proteins belong to clade VI of β-amylases, almost all members of which are predicted to be cytosolic (Thalmann *et al.*, 2019). Interestingly, *Os*BAM1 is expressed almost exclusively in seeds (Hirano *et al.*, 2011; Saika *et al.*, 2005). Using information from Ensembl Plants (https://plants.ensembl.org/Triticum_aestivum/) on shared duplications in the rice and wheat genomes (Salse *et al.*, 2008) and from the Wheat Expression Browser (expVIP: www.wheat-expression.com; Borrill *et al.*, 2016), we identified nine wheat genes putatively encoding clade VI *Os*BAM1/*At*BAM5 orthologues (Figure 6). Phylogenetic analysis suggests that these form three sets of homoeologues, namely *Ta*BAM5.1, *Ta*BAM5.2 and *Ta*BAM5.3.

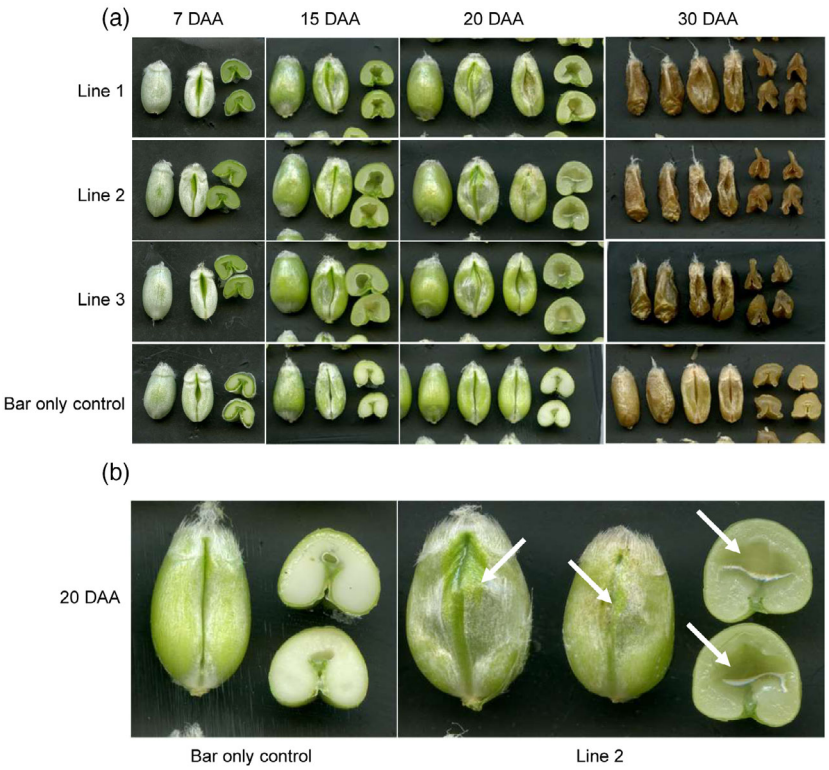

Figure 5. Appearance of grains through development.
(a) Grains at intervals through development (DAA, days after anthesis), showing views of intact grains and (right) cross-sections of grains. Note that plants in this experiment were grown in a glasshouse in summer and so reached maturity faster than those grown in a controlled environment room (Fig. 1).
(b) Close-up views of 20 DAA grains in (a). Left, bar-only control. Right, transgenic line 2 showing the liquid-filled pocket below the groove and the void above the nucellar projection in grain cross-sections. Arrows indicate the position of the pocket in intact grains, and of the void in grain cross-sections.

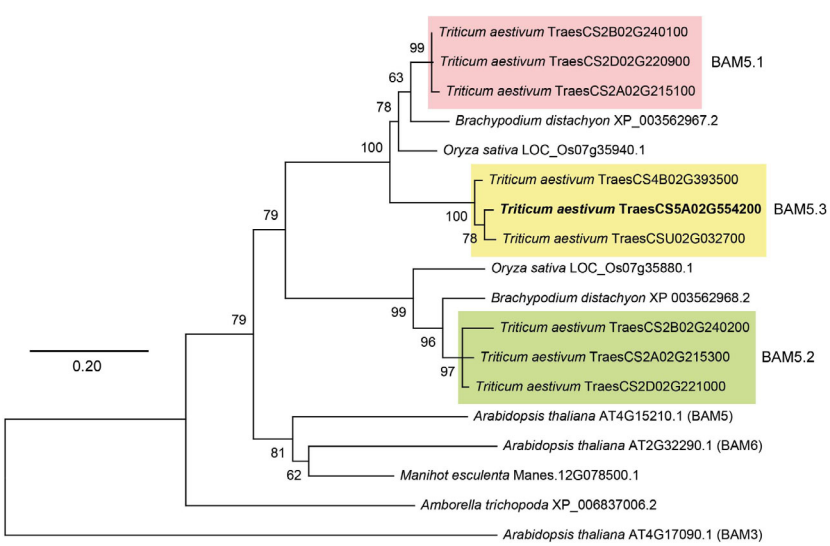

Figure 6. Phylogenetic tree of wheat BAM5 orthologues.
The tree was constructed using the maximum likelihood method with 1000 bootstrap replicates using MEGA7. Bootstrap values >50 are shown. Branch lengths represent the number of substitutions per site as indicated by the scale bar. The three sets of wheat BAM5 homoeologues (TaBAM5.1, TaBAM5.2, TaBAM5.3) are highlighted in red, green and yellow respectively. The TaBAM5.3-5A homoeologue (TraesCS5A02G554200) indicated in bold is most highly expressed in wheat endosperm during grain development (Table 1).

Expression data from expVIP indicated that expression of *Ta*BAM5.3 homoeologues is essentially confined to grains, occurring during development in the period 9–30 DAA with a possible peak at 12–14 DAA (Table 1). No expression of *Ta*BAM5.2 homoeologues was detected in grains. While *Ta*BAM5.1 homoeologues are weakly expressed in developing grains, data from expVIP indicated that expression is primarily in the seed coat, whereas *Ta*BAM5.3 is highly expressed in developing endosperm. We confirmed the cytosolic localization of *Ta*BAM5.3 by transiently expressing the protein in *N. benthamiana* leaves (Figure 7). Taken as a whole, these data are consistent with the idea that a single, cytosolic isoform of β-amylase, *Ta*BAM5.3, is expressed in developing wheat endosperm, at a point at which there is massive accumulation of maltose in transgenic grains expressing cytosolic glucan synthase.

Table 1 *Ta*BAM5 expression during grain development

	*Ta*BAM5.1			*Ta*BAM5.2			*Ta*BAM5.3		
	TraesCS2A02G 215100	TraesCS2B02G 240100	TraesCS2D02G 220900	TraesCS2A02G 215300	TraesCS2B02G 240200	TraesCS2D02G 221000	TraesCS5A02G 554200	TraesCSU02G 032700	TraesCS4B02G 393500
Leaves, 14 days (n = 5)	10	1	14	7	12	9	0	0	0
Roots, 14 days (n = 5)	1	1	14	0	0	0	0	0	4
Grain, 2 DAA (n = 2)	444	51	1171	1	6	1	0	0	0
Grain, 11 DAA(n = 5)	0	0	35	0	0	0	966	685	485
Grain, 14 DAA (n = 2)	0	0	1	0	0	0	851	965	836
Grain, 15 DAA (n = 28)	2	3	15	0	0	0	836	1009	863
Grain, 25 DAA (n = 28)	0	0	0	0	0	0	882	1128	1027
Grain, 30 DAA (n = 2)	0	0	0	0	0	0	1	1	2
Grain, 35 DAA (n = 28)	0	0	0	0	0	0	795	982	1142
Seed coat, 4 DAA (n = 1)	78	307	877	3	2	1	0	0	0
Seed coat, 8 DAA (n = 1)	26	634	1332	3	0	0	3	8	2
Seed coat, 12 DAA (n = 4)	110	114	804	0	2	0	172	73	50
Starchy endosperm, 6 DAA (1)	1	0	2	0	0	0	26	30	34
Starchy endosperm, 9 DAA (1)	0	0	1	0	0	0	897	1460	1287
Starchy endosperm, 12 DAA (3)	0	0	1	0	0	0	3127	1136	765
Starchy endosperm, 14 DAA (1)	0	0	0	0	0	0	2967	2752	2118
Starchy endosperm, 20 DAA (4)	0	0	0	0	0	0	1606	1882	1306
Starchy endosperm, 30 DAA (4)	0	0	0	0	0	0	1388	1643	1595

The three *Ta*BAM5.3 homoeologues TraesCS5A02G55420, TraesCSU02G03270 and TraesCS4B02G393500 are expressed most highly in the grain, particularly in the starchy endosperm 12–14 days after anthesis (DAA). Note that there is a well-known translocation between chr 4 and 5 that explains why the A homoeologue of BAM5.3 is on chr 5 and the B homoeologue is on chr 4. Expression data are shown as transcripts per million (tpm). Leaf and root expression data at 14 days old are shown for reference. Expression data were obtained from the public database www.wheat-expression.com.

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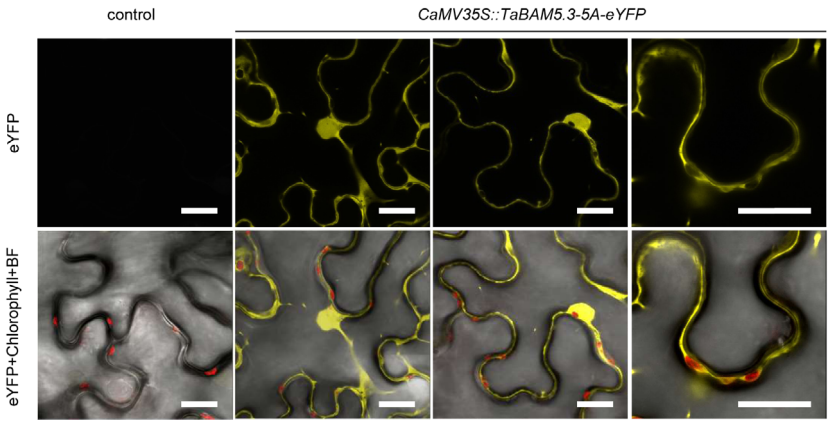

Figure 7. Cytosolic and nuclear localization of *Ta*BAM5.3-5A-eYFP in *Nicotiana benthamiana* leaves.
Full coding sequence of *Ta*BAM5.3-5A was generated by gene synthesis and inserted into the pB7YWG2 vector (with a 35S promoter and C-terminal eYFP tag; Karimi *et al.*, 2002). eYFP, enhanced yellow fluorescent protein. The construct was introduced into *N. benthamiana* leaves via *Agrobacterium* infiltration, and imaged 3 days after infiltration with confocal microscopy. Chlorophyll autofluorescence (red), eYFP (yellow) and bright field (BF). An uninfiltrated *N. benthamiana* leaf was used as control. Scale bar: 20 μm.

DISCUSSION

Despite the high activity of glycogen synthase in the cytosol in developing grains of the transgenic lines, we did not detect the presence of any novel polymeric glucans. Introduction of the *Agrobacterium* ADPGlc-dependent glucan synthase into a cellular compartment with high endogenous levels of ADPGlc might be expected to result in accumulation of linear glucans, likely to associate to form insoluble material. The absence of any such material could be attributable either to failure of the heterologous glycogen synthase to generate glucans, or their immediate degradation by endogenous cytosolic glucan hydrolases. The former explanation seems unlikely. First, we have shown previously that the *Agrobacterium* GlgA protein is active and capable of synthesizing glucans when expressed in plants [in Arabidopsis chloroplasts, directed by a transit peptide from the small subunit of Rubisco (Crumpton-Taylor *et al.*, 2013; Lu *et al.*, 2018)]. Second, it is reasonable to assume that the large amounts of maltose in transgenic lines are derived from a product of the heterologous glucan synthase. Glucan synthase activity in GlgA grain rose from 5 DAA, reaching a peak at 15 DAA and remaining high at 20 DAA, coinciding with profile reported for the high molecular weight glutenin promoter (Furtado *et al.*, 2009; Lamacchia *et al.*, 2001). Maltose levels rose very steeply from 7 to 15 DAA, and remained high at 20 DAA. Essentially no maltose was detected in control grain at any point.

We propose that a linear glucan product of glucan synthase in the cytosol is immediately hydrolysed to maltose by an endogenous β-amylase. Only one cytosolic β-amylase encoded in the wheat genome is expressed in endosperm at the time of accumulation of maltose in our transgenic plants, hence we propose that this enzyme, BAM5.3, is responsible for the conversion to maltose of the glucan synthase product.

A parallel situation occurred when UDPGlc-dependent glucan synthases were transiently expressed in *N. benthamiana* leaves (Eicke *et al.*, 2017). Maltose and maltotriose, which are present in only low amounts in untransformed leaves, were accumulated to high levels, presumably as products of metabolism of linear polymers produced by the glucan synthases.

In addition to the massive accumulation of maltose, transgenic grains differed from control grains in undergoing an almost complete cessation of starch accumulation at an early stage of development. This could be due to a depletion of the ADPGlc pool by the introduced glucan synthase, or to deleterious effects of maltose on the pathway of starch synthesis. Our results strongly favour the latter explanation. Accumulation of maltose was accompanied by low AGPase activity and the accumulation of high concentrations of sucrose in the endosperm and in the apoplast above the phloem-unloading zone. The liquid-filled pocket above the nucellar projection presumably arises because of an influx of water into the grain brought about by the apoplastic accumulation of sucrose and the leakage of maltose from endosperm cells. These features imply that maltose accumulation reduces the overall capacity for sucrose metabolism in the developing grain, limiting starch synthesis, the synthesis of other dry matter components, and ultimately its own synthesis.

Consistent with our findings, maltose accumulation has been shown previously to have strongly deleterious effects on metabolism in leaves. Arabidopsis mutants that accumulate maltose in the cytosol and/or in the chloroplast the *dpe2*, *mex1* and *be2 be3* mutants, are slow-growing and somewhat chlorotic (Dumez *et al.*, 2006; Lu *et al.*, 2006; Niittylä *et al.* (2004); Stettler *et al.*, 2009). A study of the *mex1* mutant, lacking the chloroplast envelope protein MEX1 that allows export of maltose from the chloroplast to the cytosol during starch degradation, found reduced starch turnover, high levels of chloroplast degradation and transcriptional changes consistent with accelerated protein turnover. These widespread metabolic effects were specifically caused by maltose accumulation: reducing the

accumulation of maltose by knocking out a chloroplast β-amylase in *mex1* (the *mex1 bam3* double mutant) reduced the severity of the *mex1* phenotype (Stettler *et al.*, 2009). The accumulation of maltose and maltotriose in *N. benthamiana* leaves transiently expressing a cytosolic glucan synthase also impaired normal starch turnover (Eicke *et al.*, 2017).

Although maltose accumulates to high levels around mid-development, it disappears in the latter stages of grain maturation. The strong decline in cytosolic glucan activity, plus presumably a strong limitation on ADPGlc synthesis, can explain why maltose does not accumulate further, but its disappearance implies that it can be metabolized further by enzyme(s) in the maturing grain. The nature of these activities remains unknown.

Although the introduction of glucan synthase activity into the cytosol is highly deleterious for grain development, the enzyme is clearly capable of using the cytosolic ADPGlc pool to form glucans. Two further steps that build on this finding might have the desired effect of increasing the sink strength of the grain. First, introduction of a branching enzyme (BE) as well as a glucan synthase would be expected to result in a branched cytosolic glucan that could be degraded by the endogenous β-amylase to only a limited extent. The attempts to generate cytosolic glucans by transient expression in *N. benthamiana* leaves showed that the introduction of BEs as well as glucan synthases resulted in the accumulation of soluble, branched glucan polymers to levels several times greater than normal levels of starch. The branching of the product of glucan synthases also largely suppressed the accumulation of maltose and maltotriose. We suggest that the introduction of BE as well the *GlgA*-encoded glucan synthase into the cytosol might have a similar effect, and allow glucan accumulation without deleterious effects on starch. Second, given the dramatic accumulation of maltose and its likely negative effects, removal of the cytosolic β-amylase would provide a 'clean' background for expression of glucan synthase and BE. Loss of grain β-amylase has minimal impact on seed germination in rice (Saika *et al.*, 2005) and barley (Kaneko *et al.*, 2000), hence its removal in wheat is unlikely to have strong adverse effects. Its removal could be achieved through use of the TILLING population in the Cadenza cultivar of wheat, which contains multiple lines with predicted truncations in the A, B and D genome copies of this gene (www.wheat-tilling.com; Krasileva *et al.*, 2017).

EXPERIMENTAL PROCEDURES

Plant growth and sample harvest

For initial analyses T1 plants were grown in a controlled environment room with 16 h light at approximately 400 μmol quanta m^{-2} sec^{-1} and 75% relative humidity and day/night temperatures of 20/15°C. Ears were harvested 30 days after anthesis (30 DAA: late soft dough stage under these growth conditions), immediately frozen in liquid nitrogen and then stored at −70°C. Grains for extractions were dissected from frozen ears rapidly on dry ice. Mature grains were harvested when plants had dried out. For analyses at different developmental stages, T3 plants were grown in natural light and temperature in a glasshouse in summer (July–September, day lengths 16.5 to 14 h). Ears were tagged at anthesis and harvested, stored and then dissected as above at the stated DAA. Each biological replicate was taken from a different plant.

Plant transformation was carried out as a service by the Rothamsted Research Cereal Transformation Group (https://www.rothamsted.ac.uk/cereal-transformation) using biolistics according to Sparks and Jones (2009). Construct pRRes14.3gIgA1 was bombarded into immature embryos of the wheat cultivar Cadenza alongside a selectable marker plasmid pRRes1.111, which carries a Ubi-bar-nos cassette for plant selection. Genomic DNA from all putative transgenic plants was subjected to PCR analysis for *GlgA1* (as in Crumpton-Taylor *et al.*, 2013) and the bar selectable marker gene (bar). Genotyping and copy number assessments were carried out as a service by iDNA Genetics (idnagenetics.com), on leaves of seedlings grown from grains on T0 plants. Copy number was determined by TaqMan quantitative PCR, as described by Diaz *et al.* (2012). From 30 independent transgenic lines, three with low copy number and good levels of *GlgA* expression were selected for study. A line expressing the bar gene only was selected as a control.

Quantitative reverse transcription-PCR analysis

Analysis was performed on developing grain (30 DAA) from homozygous and heterozygous T1 plants grown in a controlled environment room. Outer floret grains from the central region of a spike (approximately 60% of spike length) were pooled, giving 10–14 grains. Each spike was from a different plant. RNA extraction and cDNA synthesis were performed as described in Connorton *et al.* (2017). Briefly, RNA was extracted with phenol/chloroform extraction and then treated with DNase using the TURBODNase-free kit (Thermo Fisher, www.thermofisher.com). cDNA was synthesized using oligo(dT) primers and SuperScript II reverse transcriptase (Thermo Fisher). The ADP-ribosylation factor gene Ta2291 was used as the reference gene for quantitative reverse transcription (qRT)-PCR normalization (Vicente *et al.*, 2015).

Enzyme activities

Glucan (starch) synthase and AGPase activities were quantified on soluble extracts of 30 DAA grains sampled as for qRT-PCR analysis. Extraction (1:3 tissue to volume ratio) and assay procedures were as in Fahy *et al.* (2018). For native-gel assessment of glucan synthase activity during grain development, grains were homogenized in 100 mM MOPS, pH 7.2, 1 mM EDTA, 10% (v/v) glycerol, 1 mM dithiothreitol, and 1× Complete Protease Inhibitor cocktail (Roche, www.roche.com) at 1:3 tissue to volume ratio, then subjected to centrifugation. Soluble extract was loaded on 7.5% polyacrylamide gels containing 0.3% w/v oyster glycogen, at 4 mg tissue equivalent per lane. After electrophoresis, gels were stained with Lugol's iodine solution, according to Eicke *et al.* (2017).

Sugars, starch and glycogen

For sugar, starch and glycogen assays, frozen grains, sampled as for qRT-PCR analysis, were homogenized, extracted in perchloric acid, neutralized and subjected to centrifugation according to Fahy *et al.* (2018). Sugars were assayed on the soluble fraction, using

enzymatic assays according to Delatte *et al*. (2005) and Martinis *et al*. (2014) and an enzymatic assay for maltose described by Smirnova *et al*. (2017). Starch was assayed on the insoluble pelleted material as glucose released by treatment with α-amyloglucosidase and α-amylase, according to Smith and Zeeman (2006). Glucan was assayed as for starch, on material precipitated from the soluble fraction by treatment with 75% methanol (Delatte *et al*., 2005).

For measurements of the composition of the liquid in the pocket above the nucellar projection in GlgA grains, liquid was drawn out of five to seven grains with a fixed-needle syringe and pooled for each of the three transgenic lines. Sugars in the pooled liquid were assayed as above, with three technical replicates per line.

Dry weight and water content

Developing grains from the same ears used for metabolite measurements were weighed, dried completely at 60°C then reweighed. Water contents were calculated from these data. Mature grain size and weight were analysed with a Marvin seed analyser (www.marvitech.de).

Microscopy

Preparation of samples for scanning electron microscopy was as described by Chia *et al*. (2020).

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AUTHOR CONTRIBUTIONS

AMS designed the project; TBS, BH, LD, HS and RCG performed the experiments and contributed to specific aspects of experimental design, AMS wrote the manuscript with input and comments from all of the other authors.

CONFLICT OF INTERESTS

The authors declare that they have no competing interests.

DATA AVAILABILITY STATEMENT

All of the relevant data are presented within the manuscript.

REFERENCES

Borrill, P., Fahy, B., Smith, A.M. & Uauy, C. (2015) Wheat grain filling is limited by grain filling capacity rather than the duration of flag leaf photosynthesis: a case study using NAM RNAi plants. *PLoS One*, **10**, e0134947.

Borrill, P., Ramirez-Gonzalez, R. & Uauy, C. (2016) expVIP: A customizable RNA-seq data analysis and visualization platform. *Plant Physiology*, **170**, 2172–2186.

Cakir, B., Tuncel, A., Hwang, S.K. & Okita, T.W. (2015) Increase of grain yields by manipulating starch biosynthesis. In: Nakamura, Y. (Ed.) *Starch*. Tokyo: Springer, pp. 371–395.

Chia, T., Chirico, M., King, R., Ramirez-Gonzalez, R., Saccomanno, B., Seung, D. *et al.* (2020) A carbohydrate-binding protein, B-GRANULE CONTENT 1, influences starch granule size distribution in a dose-dependent manner in polyploid wheat. *Journal of Experimental Botany*, **71**, 105–115.

Connorton, J.M., Jones, E.R., Rodríguez-Ramiro, I., Fairweather-Tait, S., Uauy, C. & Balk, J. (2017) Wheat vacuolar iron transporter TaVIT2 transports Fe and Mn and is effective for biofortification. *Plant Physiology*, **174**, 2434–2444.

Crumpton-Taylor, M., Pike, M., Lu, K.J., Hylton, C.M., Feil, R., Eicke, S. *et al.* (2013) Starch synthase 4 is essential for coordination of starch granule formation with chloroplast division during Arabidopsis leaf expansion. *New Phytologist*, **200**, 1064–1075.

Delatte, T., Trevisan, M., Parker, M.L. & Zeeman, S.C. (2005) Arabidopsis mutants *Atisa1* and *Atisa2* have identical phenotypes and lack the same multimeric isoamylase, which influences the branch point distribution of amylopectin during starch synthesis. *The Plant Journal*, **41**, 815–830.

Diaz, A., Zikhali, M., Turner, A.S., Isaac, P. & Laurie, D.A. (2012) Copy number variation affecting the photoperiod-B1 and vernalization-A1 genes is associated with altered flowering time in wheat (*Triticum aestivum*). *PLoS One*, **7**, e33234.

Dumez, S., Wattebled, F., Dauvillee, D., Planchot, V., Ball, S.G. & D'Hulst, C. (2006) Mutants of *Arabidopsis* lacking starch branching enzyme II substitute plastidial starch synthesis by cytoplasmic maltose accumulation. *The Plant Cell*, **18**, 2694–2709.

Eicke, S., Seung, D., Egli, B., Devers, E.A. & Streb, S. (2017) Increasing the carbohydrate storage capacity of plants by engineering a glycogen-like polymer pool in the cytosol. *Metabolic Engineering*, **40**, 23–32.

Fahy, B., Siddiqui, H., David, L.C., Powers, S.J., Borrill, P., Uauy, C. *et al.* (2018) Final grain weight is not limited by the activity of key starch-synthesising enzymes during grain filling in wheat. *Journal of Experimental Botany*, **69**, 5461–5475.

Furtado, A., Henry, R.J. & Pellegrineschi, A. (2009) Analysis of promoters in transgenic barley and wheat. *Plant Biotechnology Journal*, **7**, 240–253.

Hannah, L.C., Shaw, J.R., Clancy, M.A., Georgelis, N. & Boehlein, S.K. (2017) A *brittle-2* transgene increases maize yield by acting in maternal tissues to increase seed number. *Plant Direct*, **1**, 1–9.

Hawker, J.S. & Jenner, C.F. (1993) High temperature affects the activity of enzymes in the committed pathway of starch synthesis in developing wheat endosperm. *Functional Plant Biology*, **20**, 197–209.

Hirano, T., Takahashi, Y., Fukayama, H. & Michiyama, H. (2011) Identification of two plastid-targeted β-amylases in rice. *Plant Production Science*, **14**, 318–324.

Kaneko, T., Kihara, M., Ito, K. & Takeda, K. (2000) Molecular and chemical analysis of β-amylase-less mutant barley in Tibet. *Plant Breeding*, **119**, 383–387.

Kang, G., Liu, G., Peng, X., Wei, L., Wang, C., Zhu, Y. *et al.* (2013) Increasing the starch content and grain weight of common wheat by overexpression of the cytosolic AGPase large subunit gene. *Plant Physiology and Biochemistry*, **73**, 93–98.

Karimi, M., Inzé, D. & Depicker, A. (2002) GATEWAY™ vectors for *Agrobacterium*-mediated plant transformation. *Trends in Plant Science*, **7**, 193–195.

Krasileva, K.V., Vasquez-Gross, H.A., Howell, T., Bailey, P., Paraiso, F., Clissold, L. *et al.* (2017) Uncovering hidden variation in polyploid wheat. *Proceedings of the National Academy of Sciences of the United States of America*, **114**, E913–E921.

Laby, R.J., Kim, D. & Gibson, S.I. (2001) The *ram1* mutant of Arabidopsis exhibits severely decreased β-amylase activity. *Plant Physiology*, **127**, 1798–1807.

Lamacchia, C., Shewry, P.R., Di Fonzo, N., Forsyth, J.L., Harris, N., Lazzeri, P.A. *et al.* (2001) Endosperm-specific activity of a storage protein gene promoter in transgenic wheat seed. *Journal of Experimental Botany*, **52**, 243–250.

Lu, K.J., Pfister, B., Jenny, C., Eicke, S. & Zeeman, S.C. (2018) Distinct functions of STARCH SYNTHASE 4 domains in starch granule formation. *Plant Physiology*, **176**, 566–581.

Lu, Y., Steichen, J.M., Weise, S.E. & Sharkey, T.D. (2006) Cellular and organ level localization of maltose in maltose-excess Arabidopsis mutants. *Planta*, **224**, 935–943.

Martinis, J., Glauser, G., Valimareanu, S., Stettler, M., Zeeman, S.C., Yamamoto, H. *et al.* (2014) ABC1K1/PGR6 kinase: A regulatory link between

photosynthetic activity and chloroplast metabolism. *The Plant Journal*, **77**, 269–283.

Niittylä, T., Messerli, G., Trevisan, T., Chen, J., Smith, A.M. & Zeeman, S.C. (2004) A previously unknown maltose transporter essential for starch degradation in leaves. *Science*, **303**, 87–88.

Saika, H., Nakazono, M., Akira, I., Yamaguchi, J., Masaki, S., Kanekatsu, M. *et al.* (2005) A transposon-induced spontaneous mutation results in low β-amylase content in rice. *Plant Science*, **169**, 239–244.

Salse, J., Bolot, S., Throude, M., Jouffe, V., Piegu, B., Quraishi, U.M. *et al.* (2008) Identification and characterization of shared duplications between rice and wheat provide new insight into grass genome evolution. *The Plant Cell*, **20**, 11–24.

Smidansky, E.D., Clancy, M., Meyer, F.D., Lanning, S.P., Blake, N.K., Talbert, L.E. *et al.* (2002) Enhanced ADP-glucose pyrophosphorylase activity in wheat endosperm increases seed yield. *Proceedings of the National Academy of Sciences of the United States of America*, **99**, 1724–1729.

Smirnova, J., Fernie, A.R., Spahn, C.M.T. & Steup, M. (2017) Photometric assay of maltose and maltose-forming enzyme activity by using 4-alpha-glucanotransferase (DPE2) from higher plants. *Analytical Biochemistry*, **532**, 72–82.

Smith, A.M. & Zeeman, S.C. (2006) Quantification of starch in plant tissues. *Nature Protocols*, **1**, 1342–1345.

Sparks, C.A. & Jones, H.D. (2009) Biolistics transformation of wheat. In Jones, H.D. & Shewry, P.R. (Eds.), *Methods in molecular biology Vol. 478, transgenic wheat, barley and oats*. Totowa, NJ: Humana Press, pp. 71–92.

Stettler, M., Eicke, S., Mettler, T., Messerli, G., Hörtensteiner, S. & Zeeman, S.C. (2009) Blocking the metabolism of starch breakdown products in Arabidopsis leaves triggers chloroplast degradation. *Molecular Plant*, **2**, 1233–1246.

Thalmann, M., Coiro, M., Meier, T., Wicker, T., Zeeman, S.C. & Santelia, D. (2019) The evolution of functional complexity within the β-amylase gene family in land plants. *BMC Evolutionary Biology*, **19**, 66. https://doi.org/10.1186/s12862-019-1395-2.

Tuncel, A. & Okita, T.W. (2013) Improving starch yield in cereals by over-expression of ADPglucose pyrophosphorylase: Expectations and unanticipated outcomes. *Plant Science*, **211**, 52–60.

Ugalde, J.E., Parodi, A.J. & Ugalde, J.E. (2003) De novo synthesis of bacterial glycogen: *Agrobacterium tumefaciens* glycogen synthase is involved in glucan initiation and elongation. *Proceedings of the National Academy of Sciences of the United States of America*, **100**, 10659–10663.

Vicente, R., Pérez, P., Martínez-Carrasco, R., Usadel, B., Kostadinova, S. & Morcuende, R. (2015) Quantitative RT–PCR platform to measure transcript levels of C and N metabolism-related genes in durum wheat: transcript profiles in elevated [CO_2] and high temperature at different levels of N supply. *Plant and Cell Physiology*, **56**, 1556–1573.