STARCH SYNTHASE 4 is required for normal starch granule initiation in amyloplasts of wheat endosperm

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Introduction

Starch is a major storage carbohydrate in leaves and nonphotosynthetic organs of many plants. The starch-rich endosperm of cereal grains is an important source of calories in human diets. Starch forms insoluble semi-crystalline granules that are composed of the glucose polymers – amylopectin and amylose. The biosynthesis of these polymers is relatively well understood and conserved among different plants (Smith & Zeeman, 2020). By contrast, we are only beginning to understand the mechanism of starch granule initiation, and there is vast diversity in the number and morphology of granules between different species and organs (Seung & Smith, 2019).

There are five major classes of active starch synthases – SS1, SS2, SS3, and SS4 and granule-bound starch synthase (GBSS) – which are glucosyltransferases that elongate α-1,4-linked glucan chains of starch polymers using ADP-glucose. SS1, SS2 and SS3 are involved in amylopectin synthesis, and mutants of Arabidopsis and cereals defective in these isoforms have altered amylopectin structure (Wang et al., 2003; Morell et al., 2003; Zhang et al., 2005, 2008; Delvallé et al., 2005; Fujita et al., 2006, 2007; Szydlowski et al., 2011). Amylopectin synthesis also requires branching enzymes (BEs) and debranching enzymes (isoamylases – ISAs) (Delatte et al., 2005; Dumez et al., 2006; Sundberg et al., 2013). GBSS is required for amylose synthesis (Seung, 2020). In Arabidopsis leaves, SS4 is required for both normal granule initiation and morphogenesis, but does not make a major contribution to amylopectin structure (Roldán et al., 2007; Szydlowski et al., 2009; Crumpton-Taylor et al., 2012, 2013; Seung et al., 2017; Lu et al., 2018). While chloroplasts of wild-type leaves contain multiple granules, those of the ss4 mutant typically contain only one or no granule. The granules of ss4 have distinct spherical morphology, rather than the flattened shape of wild-type starch granules. The ss4 mutant also accumulates ADP-glucose, suggesting that other SS isoforms cannot effectively utilize this substrate in the absence of SS4 (Crumpton-Taylor et al., 2013; Ragel et al., 2013).

Arabidopsis SS4 acts at least partially in complex with other proteins that are required for normal granule initiation. These include PROTEIN TARGETING TO STARCH family members, PTST2 and PTST3 (Seung et al., 2017). PTST2 is proposed to play a role in delivering maltooligosaccharide primers to SS4 for further elongation (Seung et al., 2017). SS4 interacts with a coiled-coil protein, MRC (also called PII1), but the exact role of this interaction is unknown (Seung et al., 2018; Vandromme et al., 2019).

The function of SS4 in nonphotosynthetic amyloplasts of storage organs and seeds is unknown. Granule initiation patterns in the endosperm of the Triticeae are radically different from those in Arabidopsis leaves: large, flattened A-type granules initiate

Summary

- Starch granule initiation is poorly understood at the molecular level. The glucosyltransferase, STARCH SYNTHASE 4 (SS4), plays a central role in granule initiation in Arabidopsis leaves, but its function in cereal endosperms is unknown. We investigated the role of SS4 in wheat, which has a distinct spatiotemporal pattern of granule initiation during grain development.
- We generated TILLING mutants in tetraploid wheat (Triticum turgidum) that are defective in both SS4 homoeologs. The morphology of endosperm starch was examined in developing and mature grains.
- SS4 deficiency led to severe alterations in endosperm starch granule morphology. During early grain development, while the wild-type initiated single ‘A-type’ granules per amyloplast, most amyloplasts in the mutant formed compound granules due to multiple initiations. This phenotype was similar to mutants deficient in B-GRANULE CONTENT 1 (BGC1). SS4 deficiency also reduced starch content in leaves and pollen grains.
- We propose that SS4 and BGC1 are required for the proper control of granule initiation during early grain development that leads to a single A-type granule per amyloplast. The absence of either protein results in a variable number of initiations per amyloplast and compound granule formation.

Key words: amyloplast, BGC1, endosperm, granule initiation, SS4, starch, starch synthesis, wheat.
Research

earlier during grain development, and small round B-type granules initiate 10–15 d after the A-type granules (Bechtel et al., 1990; Howard et al., 2011; Chia et al., 2020). Nonetheless, the loss of PTST2 orthologs – FLOURY ENDOSPERM 6 (FLO6) in barley and B-GRANULE CONTENT 1 (BGC1) in wheat – has major effects on granule initiation in the endosperm (Suh et al., 2004; Saito et al., 2017; Chia et al., 2020). This discovery raises the possibility that granule initiation in wheat endosperm is via an SS4-containing complex, like in Arabidopsis leaves. Here we aimed to generate and characterize wheat mutants deficient in TaSS4, to determine its role in starch synthesis in the endosperm. The mutants had highly abnormal endosperm starch morphology, resulting from the formation of compound starch granules. Interestingly, this phenotype resembled mutants defective in TaBGC1 (Chia et al., 2020). Our work demonstrates that both TaSS4 and TaBGC1 are required for the control of granule initiation in endosperm amylloplasts.

Materials and Methods

Bioinformatics analysis

TaSS4 loci (Fig. 1) were identified using BLAST against the wheat RefSeq 1.1 genome of cultivar Chinese Spring (Appels et al., 2018) on Ensemble plants (Kersey et al., 2018). TaSS4 sequences from cultivars Cadenza, Claire, Kronos, Paragon and Robigus were obtained from the Grassroots database (Clavijo et al., 2017).

TaSS4 and TaBGC1 transcript levels during tetraploid wheat grain development were extracted from the datasets of Maccaferri et al. (2019) and Xiang et al. (2019). Raw RNA-Seq reads obtained from the GenBank Sequence Read Archive (SRA) were processed using Trimmmomatic (Bolger et al., 2014) to remove adapter sequences. Processed reads were aligned to the Triticum turgidum transcriptome (Maccaferri et al., 2019) using the Quasi align mode in Salmon (Patro et al., 2017) outputting normalized expression as transcripts per million (TPM). Transcript levels of TaSS4 in different organs of hexaploid wheat were retrieved from the wheat expression database (http://www.wheat-expression.com) (Borrill et al., 2016).

Mutants in T. turgidum (cultivar Kronos) were identified using the wheat in silico TILLING resource (http://www.wheat-tilling.com) (Krasileva et al., 2017): Kronos2166 (K2166) for TaSS4-1A, Kronos2565 (K2565) and Kronos1450 (K1450) for TaSS4-1B, and Kronos2275 (K2275) for TaBGC1-4B. TaBGC1-4A mutants, Kronos2244 (K2244) and Kronos3145 (K3145) are from Chia et al. (2020). Plants were crossed to combine A- and B- homoeolog mutant alleles. AA BB, aa BB, AA bb and aa bb genotypes were selected in the F2 generation using KASP V4.0 genotyping (LGC, Teddington, UK) with primers in Supporting Information Table S1.

Wheat plants were grown in controlled environment rooms (CERs) or glasshouses at 60% relative humidity with 16 h light at 20°C and 8 h dark at 16°C. The CER light intensity was 300 μmol photons m⁻² s⁻¹. Experiments on leaves and developing grains were carried out on CER-grown material, whereas experiments with mature grains were carried out on either CER or glasshouse-grown material. Nicotiana benthamiana plants were grown in glasshouses set to provide a minimum of 16 h light at 22°C, and a dark period of 20°C. Arabidopsis thaliana plants were grown in CERs at 60% relative humidity, 12 h light (150 μmol photons m⁻² s⁻¹): 12 h dark cycles and constant temperature of 20°C.

Starch purification, granule morphology and size distribution

Endosperm starch purification: Mature grains were soaked overnight in double-distilled water (ddH₂O) at 4°C, then homogenized in a mortar and pestle with excess ddH₂O. Developing grains (stored at −80°C post-harvest) were thawed before endosperm dissection and immediately homogenized in ddH₂O using a ball mill at 30 Hz for 1 min. The homogenates were filtered (70 μm nylon mesh) then centrifuged, and the pellet was resuspended into 90% (v/v) Percoll, 50 mM Tris-HCl, pH 8 and centrifuged at 2500 × g, 5 min. The pellet was washed twice in 50 mM Tris-HCl, pH 6.8, 10 mM ethylenediaminetetraacetic acid (EDTA), 4% sodium dodecyl sulfate (SDS) (v/v), 10 mM dithiothreitol (DTT) and resuspended in ddH₂O.

Granule morphology was observed using a Nova NanoSEM 450 (FEI) scanning electron microscope. For cross-polarized light microscopy, the granules were imaged with a DFM6000 microscope fitted with a DFC 320F camera (Leica, Wetzlar, Germany). For analysis of granule size distributions, the starch was suspended into Isoton II (Beckman Coulter, Brea, CA, USA), and relative volume vs. diameter plots were generated using a Multisizer 4e Coulter counter (Beckman Coulter) with a 70 μm aperture tube. A minimum of 100,000 particles was measured per sample. All measurements were conducted with logarithmic bin spacing but are presented on a linear x-axis for clarity. The mean diameters of A- and B-type granules, and relative volume fraction of B-type granules, were calculated by fitting a mixture of two log-normal distributions in R (script available at https://github.com/JIC-CSB/coulter_counter_fitting).

Starch quantification, composition and amylopectin structure

Grain starch quantification Flour (milled in a ball mill; 5-10 mg) was dispersed in 20 μl 80% ethanol, and then incubated with 500 μl thermostable α-amylase in 100 mM sodium acetate buffer, pH 5, at 99°C for 7 min. Amyloglucosidase was added and incubated at 50°C for 35 min. All enzymes and reagents were from the Total Starch Assay kit (K-TSTA; Megazyme). The sample was centrifuged at 20,000 g for 10 min. Glucose was measured in the supernatant using the hexokinase/glucose-6-phosphate dehydrogenase assay (Roche, Basel, Switzerland), for calculation of starch content in glucose equivalents.

Leaf starch quantification 10-d-old seedlings were harvested at the base of the lowest leaf and flash frozen in liquid nitrogen (N₂). The material was homogenized in 0.7 M perchloric...
acid using a ball mill at 30 Hz. Insoluble material was pelleted by centrifugation, washed three times in 80% ethanol, then resuspended in water. Starch was digested using α-amylase/amyloglucosidase (Roche), and glucose was assayed as for grains.

Starch chain length distribution Purified starch was solubilized and enzymatically debranched using methods adapted from Wu et al. (2014), and analyzed using high-performance liquid chromatography size exclusion chromatography (HPLC-SEC) as detailed in Tuncel et al. (2019). Calibration curves were generated using pullulan standards (PSS-pulkit; Polymer Standard Service, Mainz, Germany) having peak molecular weights ranging from 342 to 708 000 Da and with correlation coefficients of $R^2 = 0.9997 \pm 0.0002$. The calibration curves were used to determine the relationship between elution volume and hydrodynamic radius ($V_h$) for the linear glucans, as described by Cave et al. (2009). The refractive index elution profiles were converted to SEC weight distributions as described by Perez-Moral et al. (2018). Amylose content was determined from chain length distributions as described by Vilaplana et al. (2012). Briefly, the cut-off between amylose and amylopectin in the chain length distribution was set at 100 degrees of polymerization (DP), and the peak areas of the amylopectin (chains $< 100$ DP) and amylose (chains $> 100$ DP) were integrated. Amylose content was estimated as the ratio of the amylopectin and amylose peak areas expressed as a percentage.

Light and transmission electron microscopy of sections

Mature grain sections After transverse grain bisection with a razor blade, thin 1 µm sections were produced from the cut surface using an Ultracut UC6 microtome (Leica) fitted with a glass knife. Sections were stained with a 1 in 20 dilution of Lugol’s iodine solution (Sigma-Aldrich, St Louis, MO, USA), and mounted in Histomount (National Diagnostics, Atlanta, GA, USA). Light microscopy was carried out on an AxioObserver Z1 microscope with an AxioCam camera (Zeiss, Jena, Germany); or a DM6000 microscope with a DFC 320F camera (Leica).

Leaf/developing grain sections Leaf segments were excised from approximately halfway along the length of a flag leaf (for wheat) or a young rosette leaf (for Arabidopsis), and fixed in 2.5% (v/v) glutaraldehyde in 0.05 M sodium cacodylate, pH 7.3 at 4°C. Developing grains (15 d post anthesis (dpa)) were cut in half before immersion in fixative. Using an EM TP embedding machine (Leica, Milton Keynes, UK), samples were post-fixed in 1% (w/v) osmium tetroxide (OsO4) in 0.05 M sodium cacodylate for 2 h at room temperature, dehydrated in ethanol and infiltrated with LR White resin (Agar Scientific, Stansted, UK). LR White blocks were polymerized at 60°C for 16 h. For light microscopy, the semi-thin sections (0.5 µm) were prepared. Leaf sections were stained with reagents from the Periodic Acid Schiff kit (ab150680; Abcam), by incubating 30 min in the periodic acid solution and 5 min in Schiff’s reagent, then staining with

Fig. 1 Schematic illustrations of wheat TaSS4 homoeologs. (a) Location of TaSS4 homoeologs on group 1 chromosomes. The red boxes represent TaSS4 homoeologs, while homoeologs of the adjacent genes are shown in green (phosphodiesterase-like protein), purple (glycoside hydrolase family 18 protein), blue (P loop-containing nucleoside triphosphate hydrolase) and orange (serine acetyltransferase-like protein). Arrowheads on the boxes indicate direction of transcription. Chromosome coordinates are indicated below each region. (b) Gene models of the TaSS4 homoeologs. Exons are represented with blue boxes, while light blue boxes represent the 5’ and 3’ untranslated regions (UTRs). Mutations in the Tass4-1 and Tass4-2 mutant lines are depicted with red arrows and the mutated codons/ amino acids are shown in red letters. The polymorphism in TaSS4-1A between Kronos and Chinese Spring (CS) is indicated.
1% (w/v) toluidine blue for 30 s before mounting in Histomount. Sections from developing grains were stained with 1% (w/v) toluidine blue. Light microscopy was carried out as described earlier. For transmission electron microscopy (TEM), ultrathin sections (~80 nm) were cut with a diamond knife and placed on formvar and carbon-coated copper grids (EM Resolutions, Sheffield, UK). The sections were stained with 2% (w/v) uranyl acetate for 1 h and 1% (w/v) lead citrate for 1 min, washed in distilled water and air dried. Sections were viewed on a Talos 200C TEM (FEI) at 200 kV and imaged with a OneView 4K × 4K camera (Gatan, Warrendale, PA, USA).

Visualization and scoring of starch in pollen

Mature anthers were harvested at 80% (v/v) ethanol and stained with a 1 in 20 dilution of Lugol’s iodine solution (Sigma-Aldrich) overnight. After destaining in ddH2O, pollen was observed with light microscopy as described earlier. The percentage of starchless pollen (no visible iodine stain) was calculated by scoring the first c. 100 pollen grains observed.

Cloning and transformation of plant material

TaSS4-1A, TaSS4-1B, TaBGC1-4A and TaBGC1-4B coding sequences were codon optimized to ease sequence complexity and synthesized as gBlocks gene fragments (IDT DNA), flanked with attB1 and attB2 Gateway recombination sites. The optimized sequences are provided in Table S2. The fragment was recombined into the pDONR221 vector using BP Clonase II (ThermoFisher Scientific, Waltham, MA, USA). The sequences were recombined at attB1 and attB2 Gateway recombination sites. The optimized sequences are provided in Table S2. The fragment was recombined into the pDONR221 vector using BP Clonase II (ThermoFisher Scientific, Waltham, MA, USA). The sequences were recombined into pUBC-YFP (Ubiquitin10-driven expression and C-terminal YFP-tag) (Greffen et al., 2010) or pJC52 (CaMV 35S-driven expression and C-terminal HA-tag) (Karimi et al., 2002).

For transient expression in N. benthamiana, Agrobacterium tumefaciens (strain AGL-1 or GV3101) harboring the relevant constructs were grown at 28°C overnight. After destaining in ddH2O, pollen was stained with a 1 in 20 dilution of Lugol’s iodine solution (Sigma-Merck). Mature anthers were harvested into 80% (v/v) ethanol and air-dried. Sections were viewed on a Talos 200C TEM (FEI) at 200 kV and imaged with a OneView 4K × 4K camera (Gatan, Warrendale, PA, USA).

Results

Mutants lacking TaSS4 produce aberrant endosperm starch

Hexaploid wheat has three homoeologs of TaSS4 on group 1 chromosomes (Irshad et al., 2019). The B- and D-genome homoeologs were reported to have 16 exons and the A-genome homoeolog has 13 exons. We established that in the most recent wheat genome release (RefSeq v.1.1 cv Chinese Spring; Appels et al., 2018), these homoeologs correspond to TaSS4-1A (TaeCS1A02G353300), TaSS4-1B (TaeCS1B02G368500) and TaSS4-1D (TaeCS1D02G356900) (Fig. 1a). As reported by Irshad et al. (2019), TaSS4-1B and TaSS4-1D loci contained 16 exons (Fig. 1b) but TaSS4-1A had a shorter coding sequence generated from 13 exons. Nonetheless, the predicted transcript length of TaSS4-1A was the same as the other homoeologs because it had a longer 5′ UTR region. To investigate the discrepancy in gene model between homoeologs, we compared nucleotide and predicted amino acid sequences from the Chinese Spring reference sequence with those of other sequenced hexaploid wheat cultivars (Cadena, Paragon, Robigus, Claire) and the tetraploid cultivar Kronos on the Grassroots database (Clavijo et al., 2017) (Supporting Information Fig. S1a). For all cultivars except for Chinese Spring, TaSS4-1A was predicted to have all 16 coding exons. Chinese Spring had a unique single nucleotide polymorphism (SNP) that results in a premature stop codon in a position occupied by exon 4 in the gene model for the other cultivars (Figs 1b, S1a). This SNP most likely led to the incorrect prediction of 13 coding exons and a long 5′UTR for TaSS4-1A in the Chinese Spring sequence.
To assess the importance of TaSS4 in endosperm starch formation, we created mutants of tetraploid wheat defective in both homoeologs of TaSS4. We used the collection of exome-capture sequenced, EMS-mutagenized TILLING lines of the tetraploid wheat Kronos (Krasileva et al., 2017; http://www.wheat-tilling.com) to identify mutants that are likely to have no TaSS4-1A or TaSS4-1B protein. The predicted amino acid sequences of TaSS4-1A and TaSS4-1B from Kronos shared 99–100% identity with those from the Chinese Spring reference genome, and the two Kronos homoeologs were 97% identical to each other (Fig. S1a,b). For TaSS4-1A, we obtained the Kronos2166 (K2166) line, which carries a splice donor site mutation after exon 5 (Fig. 1b). For TaSS4-1B, we obtained Kronos2565 (K2565) carrying a premature stop codon in place of Trp364, and Kronos1450 (K1450) carrying a splice acceptor site mutation before exon 15. The presence of each mutation was confirmed by KASP genotyping using the primers in Table S1. The K2166 line was crossed with K2565 to create the Tass4-1 lines, or with K1450 to create the Tass4-2 lines. KASP genotyping was used to identify F1 or F2 individuals homozygous for both A and B mutations (aa bb), homozygous for only the TaSS4-1A mutation (aa BB) or the TaSS4-1B mutation (AA bb), and ‘negative segregant’ controls that lacked both mutations (AA BB). Except where specified, observations below were on the Tass4-1 lines.

To observe TaSS4 protein levels during grain development and the effect of the Tass4-1 mutations on TaSS4 protein abundance, we generated an antiserum against a TaSS4-1B recombinant protein, expressed in and purified from E. coli. Immunoblots of TaSS4-1A and TaSS4-1B proteins transiently expressed in N. benthamiana leaves demonstrated that the antiserum recognized both homoeologs (Fig. S2). Protein extracts from endosperms dissected from wild-type developing grains (10, 15 and 20 dpa) were immunoblotted with the antiserum. A band that corresponded to the predicted size of the mature polypeptide (98 kDa) was observed at all three timepoints, but was most prominent at the 10 dpa timepoint (Fig. 2a). Several other bands at different molecular weights were detected, but comparison of immunoblots from the wild-type and mutant extracts showed that the 98 kDa band was missing in the latter while other bands were unaffected (Fig. 2b).

We conclude that the 98 kDa band represents TaSS4, and that the other bands result from nonspecific binding.

Transcript data for whole caryopses (Maccaferri et al., 2019) and dissected endosperm (Xiang et al., 2019) from developing tetraploid wheat grains revealed that TaSS4 transcript levels were higher at the early stages of grain development (8–11 dpa) than later stages (16–22 dpa) (Fig. S3). These data are consistent with the observed decrease in TaSS4 protein levels at later stages of grain development (Fig. 2a).

To assess the impact of the Tass4-1 mutations on endosperm starch, we purified starch granules from mature grains and observed them using scanning electron microscopy. Granules from control lines (AA BB) and single homoeolog mutants (aa BB and AA bb) had flattened A-type granules and round B-type granules typical of wheat starch (Fig. 3a). By contrast, most starch granules from the double mutant (aa bb) had irregular, polyhedral morphology. The irregular granules were highly variable in size, but rarely exceeded that of a typical A-type granule. A-type granules of normal appearance were also present in the double mutant, but we rarely observed normal B-type granules.

We used cross-polarized light microscopy to examine the origins of the larger polyhedral granules in the Tass4-1 double mutant endosperm. In the control line and single homoeolog mutants, there was one ‘Maltese cross’ per A-type or B-type granule, indicating a single center of organization (Fig. 3b). The few normal A-type granules in the double mutant also had single crosses. However, a complex birefringence pattern with faint or multiple crosses were observed in most polyhedral granules, indicating multiple initiation points.

Using a Coulter counter, we examined the granule size distribution in the endosperm starches. As expected, starch from the

![Fig. 2 TaSS4 and TabGC1 protein levels in developing wheat endosperm. (a) Total proteins were extracted from developing endosperms at 10, 15 and 20 d post anthesis (dpa), and immunoblotted using anti-TaSS4 (upper panel), anti-TabGC1 (middle panel) or anti-actin (lower panel) antibodies. Lanes were loaded on an equal protein basis. The migration of molecular weight markers are indicated in kilodaltons (kDa) to the left of each panel. Two replicate extractions for each genotype (numbered 1 and 2) were prepared from grains harvested from two different plants. (b) Same as (a), but with Tass4-1 grains harvested at 10 dpa. (c) Same as (a), but with Tabgc1-1 grains harvested at 20 dpa.](image-url)
control line and single mutants showed a bimodal size distribution, with peaks at c. 20 µm diameter for A-type and 7–8 µm diameter for B-type granules (Fig. 3c). The size and relative proportion (by volume) of A-type and B-type granules were quantified by fitting a mixed log-normal distribution (Table 1; Tanaka et al., 2017). There were no significant differences between the control and single mutants. The granule size distribution of the double mutant had no distinct peaks, and neither a mixed nor a single distribution could be fitted reliably to these data.

The normal granule morphology of control (AA BB) and single mutant (aa BB and AA bb) lines indicates that the aberrant morphology arises only when both Tass4 homoeologs are defective (aa bb). However, it remained possible that the aberrant morphology arose from a combination of background mutations in the single-mutant parents of the double mutant. To exclude this, we first backcrossed the double mutant to the wild-type in the single-mutant parents of the double mutant. To exclude morphology arose from a combination of background mutations (Tass4 et al., 2019). The chain length distribution of amylopectin and amylose, and the estimated amylose content, were identical in control and mutant starches (Fig. S6; Table 1). Thus, the altered granule morphology in the Tass4-1 double mutant cannot be attributed to differences in starch content, composition, or structure. The starch content of mature grains was not significantly different on a dry weight basis between control, single and double mutant lines (Table 1). To examine amylopectin/amyllose structure and abundance, debranched starch was subjected to HPLC-SEC with refractive index detection (Cave et al., 2009; Tuncel et al., 2019). The chain length distribution of amylopectin and amylose, and the estimated amylose content, were identical in control and mutant starches (Fig. S6; Table 1). Thus, the altered granule morphology in the Tass4-1 double mutant cannot be attributed to differences in starch content, composition, or polymer structure.

TaSS4 mutations do not alter total starch content, composition or amylopectin structure

We investigated whether the aberrant granule morphology in the Tass4-1 aa bb line was accompanied by changes in starch content, composition or structure. The starch content of mature grains was not significantly different on a dry weight basis between control, single and double mutant lines (Table 1). To examine amylopectin/amyllose structure and abundance, debranched starch was subjected to HPLC-SEC with refractive index detection (Cave et al., 2009; Tuncel et al., 2019). The chain length distribution of amylopectin and amylose, and the estimated amylose content, were identical in control and mutant starches (Fig. S6; Table 1). Thus, the altered granule morphology in the Tass4-1 double mutant cannot be attributed to differences in starch content, composition, or polymer structure.

Table 1 Starch content, composition and granule size in Tass4-1 mature grains.

| Genotype | Starch content (% flour weight) | Amylose content (% starch) | A-type granule mean diameter (µm) | B-type granule mean diameter (µm) | B-type granule volume (%) |
|----------|-------------------------------|--------------------------|----------------------------------|----------------------------------|--------------------------|
| AA BB    | 50 ± 3                        | 31 ± 2                   | 19.1 ± 0.7                       | 7.9 ± 0.9                        | 38.1 ± 2.2               |
| aa BB    | 59 ± 4                        | 32 ± 2                   | 18.9 ± 0.4                       | 6.7 ± 0.1                        | 39.8 ± 0.8               |
| AA      | 51 ± 5                        | 32 ± 3                   | 20.5 ± 0.3                       | 7.4 ± 0.1                        | 37.9 ± 0.8               |
| AA bb   | 42 ± 2                        | 30 ± 1                   | 20.2 ± 0.4                       | 7.5 ± 0.1                        | 38.5 ± 2.2               |
| AA         |                                           |                    |                                              |                                  |                         |

Starch content was determined as glucose equivalents and is expressed as a percentage of the flour weight. Amylose content of starch was determined by high-performance liquid chromatography size exclusion chromatography (HPLC-SEC). The mean diameters of A-type and B-type granules and the relative volume of B-type granules were determined using a Coulter counter. All values are mean ± SE from n = 3 biological replicates, defined as grains harvested from three different plants. There were no significant differences between any of the lines in any of these parameters under a one-way analysis of variance (ANOVA) at P < 0.05.
The **Tass4** mutant shows defective granule morphology during early grain development

To investigate at which stage of grain development the aberrant granules in the **Tass4-1** double mutant form, we examined granule morphology and size distribution of starch extracted from developing endosperms at three different time points: 8, 14 and 21 dpa. As expected, the wild-type endosperm contained only A-type granules at 8 dpa, with a peak at c. 15 µm diameter (Fig. 4a, b). B-type granules were present at 14 and 21 dpa, with a peak at c. 5 µm diameter. Starch from the **Tass4-1** double mutant already contained aberrant, polyhedral granules at 8 dpa, and no distinct A-type and B-type granule peaks were observed in the mutant at any timepoint.

The **Tass4** mutant produces compound granules

We examined the spatial arrangement of the polyhedral granules within the endosperm of the **Tass4-1** double mutant, initially in thin sections of mature grains stained with iodine solution. Consistent with the observations made on purified starch, the control and single mutant lines had normal A- and B-type granules. The granules with polyhedral shapes in the double mutant were almost always tessellated within larger structures (Fig. 5).

Observing sections of developing endosperm at 15 dpa by light microscopy and TEM revealed remarkable heterogeneity among amyloplasts in the mutant (Fig. 6). Whereas the amyloplasts in the wild-type contained single A-type granules and some peripheral stroma, most amyloplasts in the mutant contained compound granules, while some contained A-type granules that were indistinguishable from those of the wild-type – and in most endosperm cells, both types of amyloplasts were present. The number of individual ‘granulae’ visible within each compound granule section varied, ranging from 4 to > 40. Notably, some amyloplasts had formed granules that were tessellated in tubular structures.

**Tass4** starch granules resemble those of the **Tabgc1** mutant

The elimination of another component of the putative granule initiation complex defined in Arabidopsis, PTST2/FLO6/BGC1, results in strong granule morphology defects in endosperms of barley and hexaploid wheat, including the occurrence of ‘semi-compound’ granules (Suh et al., 2004; Chia et al., 2020). To discover the relationship between the roles of **TaSS4** and **TaBGC1** in wheat endosperm, we compared phenotypes of **Tass4** and **Tabgc1** mutants in the same Kronos background. For two **Tabgc1 aa bb** double mutants (Fig. 7a) that accumulate no detectable **TaBGC1** protein (Fig. 2c), we established that starch
granules had morphologies like those described for Tabgc1 mutants in hexaploid wheat and barley: mature grains contained A-type granules of normal appearance and small polyhedral granules (Fig. 7b). As in the Tass4 mutant, these polyhedral granules were already present during early grain development (8 dpa onwards) (Fig. S7). However, normal A-type granules were more frequent in the Tabgc1 mutant (Fig. 7b) than in the Tass4 mutant (Fig. 3a). Coulter counter analysis also showed a prominent A-type granule peak in the Tabgc1 mutant at a similar diameter to that of the wild-type (Fig. 7c,d). Such a distinct peak was not observed in the Tass4-1 mutant (Fig. 3c).

Fig. 5 Endosperm sections of Tass4-1 single and double mutants. (a) Thin sections were prepared from mature wheat grains, stained with iodine and observed using light microscopy. Single (aa BB or AA bb) and double mutants (aa bb) were compared with control (AA BB) lines. Bars, 25 µm. (b) Insets showing a close-up view of a large compound structure (left panel) and a tubule-like structure (right panel) in the aa bb section – both indicated with red arrows. Bars, 25 µm.

Fig. 6 Compound granules in the developing Tass4-1 endosperm. (a) Toluidine blue-stained sections of developing wheat endosperm (15 d post anthesis (dpa)) in the Tass4-1 double mutant (aa bb) or control (AA BB), observed using light microscopy. Blue arrows indicate examples of normal A-type granules, while red arrows indicate compound granules. Bars, 20 µm. (b) Same as (a), but observed using transmission electron microscopy. Amyloplast membranes and stromal space around granules are indicated with yellow arrows. Bars, 5 µm.
Loss of TaSS4 affects starch synthesis in pollen

The Tass4 double mutant was indistinguishable from control lines in terms of plant growth (Fig. 8a). Most grains of the mutant appeared normal and the average weight of individual grains was not significantly altered compared to the wild-type, although we observed rare examples of smaller, shrivelled grains in the mutant (Figs 8b, S8). While the double mutant produced comparable numbers of tillers to the control (Fig. 8c), the number of grains per spike was significantly reduced in the mutant (Fig. 8d). This reduction in grain number was most severe in the nonbackcrossed Tass4-1 double mutant but was partly recovered after backcrossing, suggesting this phenotype was exacerbated by background mutations in nonbackcrossed lines (Fig. 8d). Since the fewer grains in the backcrossed mutant suggests defective fertilization, we examined starch accumulation in pollen grains of the mutant using iodine staining. Less than a third of pollen grains from the double mutant contained starch, contrasting those from control lines where almost all contained starch (Figs 8e, S9a). Cross-pollination experiments with the backcrossed Tass4-1 lines demonstrated that using aa bb pollen to fertilize AA BB maternal plants resulted in significantly reduced fertilization rates compared to the reciprocal cross (Fig. S9b). We also observed fewer than expected aa bb mutants segregating from aa Bb individuals of the BC2F2 generation (Table S3). These data suggest that TaSS4 is important for normal pollen starch synthesis and viability. Interestingly, grains from low-yielding nonbackcrossed and high-yielding backcrossed Tass4-1 lines had identical starch granule morphology (Fig. S5), demonstrating that this phenotype is independent from the fertility phenotype.

Loss of TaSS4 results in fewer starch granules per leaf chloroplast

Since SS4 plays a critical role in granule initiation and morphogenesis in Arabidopsis leaves, we investigated whether these roles are conserved in wheat leaves. Leaves of the Tass4-1 double mutant accumulated less than half the starch content of the control over the light period (Fig. 9a). Light microscopy to visualize granules in chloroplasts at the end of the day showed similar frequency distributions of granule sections per chloroplast section in the control and single mutant lines: 70 to 80% of chloroplasts contained between one and eight granule sections and the remainder contained no visible starch granule (Fig. 9b,c). By contrast, almost 80% of chloroplasts in the double mutant contained...
no visible starch granule. Examination with TEM showed that granules in control leaves had the typical flattened shape of leaf starch, whereas most granules in the double mutant were small and rounded (Fig. 9d).

These results suggest that as in Arabidopsis, the loss of TaSS4 in wheat strongly affects the number of granules initiated per chloroplast. We therefore attempted to complement the Arabidopsis Atss4 mutant by expression of TaSS4-1B with a C-terminal YFP tag and under the Arabidopsis Ubiquitin 10 promoter (pUBQ). The Atss4-1 mutant had pale leaves, but the transformed lines were not pale (Fig. 10a). The transformed lines had multiple granules in most chloroplasts, whereas most chloroplasts of the Atss4-1 mutant were either starchless or contained a single large, round granule (Fig. 10b). TaSS4 can thus partially complement the granule number phenotype of the Atss4 mutant. Most granules in the transgenic lines were irregularly shaped, and few were flattened as in the wild-type, or round as in Atss4 (Fig. 10c) – suggesting TaSS4 can also influence granule morphology when expressed in Arabidopsis leaves.

Discussion

TaSS4 is necessary for normal granule initiation in the endosperm

In wheat endosperm, granule initiation is spatially and temporally coordinated such that single A-type granules form in amyloplasts during early grain development and B-type granules initiate later and at least partially in stroma-filled tubules (stromules) that emanate from the amyloplast (Parker, 1985;
This pattern is distinct from most other grasses (e.g. rice), which form compound granules by initiating multiple granules per amyloplast during early grain development (Matsushima et al., 2013, 2015). Recent work in Arabidopsis leaves has suggested a mechanism of granule initiation in leaf chloroplasts involving at least six proteins – SS4, SS5, PTST2, PTST3, MFP1 and MRC, each of which is individually necessary for normal granule initiation (Seung & Smith, 2019; Abt & Zeeman, 2020). Among these initiation proteins, only SS4 is known to have enzymatic activity (Roldán et al., 2007; Szydlowski et al., 2009; Abt et al., 2020). However, the influence of SS4 on the distinct granule initiation patterns observed in cereal amyloplasts was not known. Our study demonstrates that TaSS4 is required for the control of granule initiation in wheat endosperm. Loss of TaSS4 in wheat did not affect the content, composition or polymer structure of endosperm starch (Table 1), but resulted in the formation of compound granules in the endosperm in place of most A-type granules (Figs 3–6). A similar phenotype was observed in mutants fully deficient in TaBGC1 in tetraploid wheat (Fig. 7), and in hexaploid wheat (Chia et al., 2020), suggesting the two proteins act in a similar process. However, the Tass4 phenotype was more severe than the Tabgc1 phenotype as there were substantially more normal A-type granules in the latter (Fig. 7). These observations parallel those in Arabidopsis leaves, in which granule initiation is more compromised in the Atss4 mutant than in the Atpts2 mutant (Seung et al., 2017).

To our knowledge, our work provides the first demonstration that SS4 plays a major role in granule initiation in amyloplasts of...
cereal grains. The severe defects in granule initiation in the *Tass4* mutant contrasts with the minor defects in compound starch granule morphology in the rice *OSS4b* mutant (Toyosawa et al., 2016). However, rice has two *SS4* paralogs, and the extent to which the other paralog (*OSS4a*) can compensate for the loss of *OSS4b* in the endosperm is unknown. Interestingly, *OSS4a* knockout mutants created by gene-editing were observed to have severe defects in plant growth (Jung et al., 2018). Examining endosperm starch in these mutants, as well as in an *oss4a oss4b* double mutant, will be informative of the role of *SS4* in a species that already produces compound granules.

**How do TaSS4 and TaBGC1 control the number of granule initiations?**

The increase in initiations per amyloplast that leads to compound granule formation following loss of *SS4* in wheat endosperm contrasts with the reductions in granule number per chloroplast observed in both Arabidopsis and wheat leaves (Roldán/C19 et al., 2007) (Fig. 9). Thus, in wheat endosperm, neither TaSS4 nor TaBGC1 is strictly required for the initiation of granules per se, but both are required to control the process—such that single *A*-type granules initiate in amyloplasts during early grain development. It remains to be determined how these proteins exert this control. It is possible that TaSS4 and TaBGC1 together form a single granule initiation per amyloplast, from which the other enzymes of starch biosynthesis can build a single *A*-type granule (Fig. 11). The formation of this single granule initiation may be enough to suppress the formation of more granules—since the activity of other starch biosynthesis enzymes can be directed towards the growing granule. However, in the absence of this single granule initiation, the other enzymes may start elongating any available substrate, such as soluble maltooligosaccharides, leading...
to an uncontrolled number of granules being initiated. These enzymes may include starch synthases and starch phosphorylase, which can all elongate maltooligosaccharides in vitro (Hwang et al., 2010; Brust et al., 2013; Cuesta-Seijo et al., 2016). The heterogeneity in granule number among amyloplasts in the endosperm of \(TaSS4\) and \(Tabgc1\) mutants may reflect stochasticity in the number of initiations per amyloplast that occur in the absence of \(SS4\) or \(BGC1\). It is also possible that some amyloplasts fail to initiate starch granules, but it is difficult to distinguish empty amyloplasts from other membranous structures in TEM images of the endosperm.

It is unknown which features of \(TaSS4\) allow it to initiate a single granule per amyloplast. Notably, distinct patterns of protein localization have been observed for \(AtSS4\) in Arabidopsis leaves, and for \(OsSS4b\) in rice amyloplasts – where it locates to the septum-like structures of compound granules (Toyosawa et al., 2016). We are currently exploring the localization of \(TaSS4\) in amyloplasts of developing grains and whether that could explain a single point of A-type granule initiation. Since granule initiation proteins in Arabidopsis leaves act via protein–protein interactions, searching for interacting proteins may also provide insight on how \(TaSS4\) and \(TaBGC1\) act in wheat endosperm. \(AtSS4\) is proposed to interact with \(APST2\) in Arabidopsis leaves (Seung et al., 2017). Although we attempted multiple co-immunoprecipitation and pulldown approaches, we failed to find any evidence that \(TaSS4\) and \(TaBGC1\) interact in the endosperm (data not shown). Further work is required to determine if they interact only weakly or transiently. Possible interactions of these proteins with \(ISOAMYLASE 1\) (\(ISA1\)) should also be investigated since \(ISA1\) is reported to interact with \(PTST2\) (\(FLO6\)) in rice (Peng et al., 2014). Notably, \(isa1\) mutants of barley contain compound granules that resemble those of \(TaSS4\) mutants (Burton et al., 2002), providing a strong indication for \(ISA1\) involvement in granule initiation.

The specific role of \(TaSS4\) in B-type granule initiation must also be further explored. Very few normal round B-type granules were observed in mature grains of the \(TaSS4\) mutant (Fig. 3). Also, at 15 dpa, we observed many compound granules in a linear arrangement in the mutant, raising the possibility that they formed in stromules that normally enclose B-type granules (Fig. 6). Interesting, Chia et al. (2020) reported that reducing gene dosage of \(TaBGC1\) in hexaploid wheat can almost eliminate B-type granules while retaining normal A-type granule morphology. By contrast, B-type granule volume was not affected in either of the single homoeolog mutants in \(TaSS4\), but it is possible that a further reduction in gene dosage is required to see an effect. However, we noted that while \(TaSS4\) protein levels are highest during early grain development and decrease at the later developmental stages, \(TaBGC1\) transcript and protein levels increase and are highest during the period of B-type granule initiation (Figs 2, S3). Thus, it is possible that \(TaBGC1\) has a specific role during B-type granule initiation that is independent of \(TaSS4\).

While other members of the Triticeae (e.g. barley and rye) also have A- and B-type granules, most other grasses produce compound granules in the endosperm (Matsushima et al., 2013, 2015). The fact that loss of \(SS4\) or \(BGC1\) gives rise to some compound granules in wheat makes it tempting to speculate that differences in the extent and timing of \(SS4\) and/or \(BGC1\) expression between species could determine whether a given species possesses compound granules. However, the difference between compound and other patterns of granule initiation is unlikely to be so simple. Compound granules of rice have complex structural features, including membranes and septum-like structures that separate each constituent granula (Yun & Kawagoe, 2010; Kawagoe, 2013; Toyosawa et al., 2016). Thus, formation of compound granules in rice is likely to involve multiple genes that control starch synthesis and amyloplast morphogenesis.

\(TaSS4\) is required for proper granule initiation in leaves and pollen

In leaves of the Arabidopsis \(Atss4\) mutant, over 75% of chloroplasts had no visible starch granule, and the majority of remaining chloroplasts contained one large granule (Roldán et al., 2007; Seung et al., 2017). Leaves of the \(TaSS4\) mutant had a percentage of starchless chloroplasts comparable to the Arabidopsis mutant, but the remaining chloroplasts mostly contained multiple granules (Fig. 9). The reason for this difference between the Arabidopsis and wheat phenotypes is unknown, but could reflect differences in the compensation mechanism following loss of \(SS4\). The few granules present in the Arabidopsis \(Atss4\) mutant are likely initiated by \(SS3\), since the \(AtSS3\) \(AtSS4\) double mutant is almost starchless (Szyladowski et al., 2009; Seung et al., 2016). Further work is required to determine whether \(SS3\) initiates the starch granules in leaves of the \(TaSS4\) mutant.

The expression of \(TaSS4\), which shares 56% amino acid sequence identity with \(AtSS4\) (BLAST pairwise alignment), could largely restore the initiation of multiple granules per chloroplast when expressed in the Arabidopsis \(Atss4\) mutant. However, the exact role of \(TaSS4\) in granule morphogenesis in leaves remains unclear. Starch granules in the \(TaSS4\) mutant were small and round (Fig. 9), but distinct from the large, rounded granules of the \(Atss4\) mutant (Fig. 10). \(TaSS4\) expression in the \(Atss4\) mutant resulted in aberrant granule morphology, which was distinct from both the round granules of \(Atss4\) and the flattened granules of the wild-type. These aberrant granule shapes may result from partial complementation by \(TaSS4\) that achieves an ‘intermediate’ morphology between round and flattened, or abnormal function of \(TaSS4\) in Arabidopsis leaves (e.g. due to missing interaction partners or other regulatory factors).

Despite a reduction in gene dosage to 50% in our single homoeolog wheat mutants, we did not observe an effect on granule number in leaf chloroplasts. On first glance, this is in contrast to a previous report that hexaploid wheat mutants deficient in only \(TaSS4-1D\) have reduced numbers of granules per chloroplast (Guo et al., 2017). However, we showed that some hexaploid cultivars, including the reference cultivar Chinese Spring, have a natural polymorphism that leads to a premature stop codon in \(TaSS4-1A\) (Fig. 1). It is possible that \(TaSS4-1B\) is the only functional homoeolog in the \(TaSS4-1D\) mutants of Guo et al. (2017) (in cultivar Jing411), and thus may have a functional gene dosage of only 33%.
TaSS4 also appears to be required for normal starch synthesis in wheat pollen. Publicly available gene expression data for hexaploid wheat suggests that TaSS4 is expressed in microspores in addition to leaves, stems, roots and grains (Fig. S3b); and most pollen grains from our TaSS4 mutants were starchless (Figs 8c, S9). In rice, starch synthesis in pollen appears to be essential for viability, as rice pgm mutants lacking pollen starch are sterile (Lee et al., 2016). Consistent with this, the pollen from the TaSS4 mutant had significantly reduced fertilization success in cross-fertilization experiments (Fig. S9b), and the mutants produced fewer grains per spike (Figs 8d, S9c). These grains likely result from the small proportion of mutant pollen that contains starch. Further work should examine the effects on granule number and morphology in these starch-containing pollen grains.

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Author contributions

EH and DS conceived and led the study and designed most of the experiments. EH conducted most of the experiments. JC designed and conducted the Arabidopsis complementation experiments. AW-L designed and conducted the transcriptomics analysis. JA-J and FJW designed and conducted the HPLC-SEC analysis. JEB designed and conducted TEM experiments and performed sectioning. BF designed and conducted the crosses of the wheat TILLING lines. MH designed the analysis of the granule size distribution data. All authors analyzed data. EH and DS wrote the article with contributions from all authors.

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Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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### Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

**Fig. S1** Amino acid sequence alignment of the wheat TaSS4 homoeologs.

**Fig. S2** TaSS4 and TaBGC1 antibodies can detect both A- and B-genome homoeologs.

**Fig. S3** Expression analysis of TaSS4 and TaBGC1.

**Fig. S4** Backcrossing scheme for Tass4-1.

**Fig. S5** Granule morphology of Tass4-1 after backcrossing and Tass4-2.

**Fig. S6** Chain length distribution of debranched Tass4-1 starch.

**Fig. S7** Starch from developing grains of Tabgc1.

**Fig. S8** Average grain weight of Tass4 mutants.

**Fig. S9** Starch in pollen grains of Tass4 mutants.

**Table S1** Oligonucleotides used in this study.

**Table S2** Codon-optimized coding sequences for TaSS4 and TaBGC1.

**Table S3** Segregation of the Tass4-1 aa bb genotype from BC2F2 aa Bb individuals.

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