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RESEARCH PAPER

Waxy and non-waxy barley cultivars exhibit differences in the targeting and catalytic activity of GBSS1a

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Abstract

Amylose synthesis is strictly associated with activity of granule-bound starch synthase (GBSS) enzymes. Among several crops there are cultivars containing starch types with either little or no amylose known as near-waxy or waxy. This (near) amylose-free phenotype is associated with a single locus (waxy) which has been mapped to GBSS-type genes in different crops. Most waxy varieties are a result of either low or no expression of a GBSS gene. However, there are some waxy cultivars where the GBSS enzymes are expressed normally. For these types, single nucleotide polymorphisms have been hypothesized to represent amino-acid substitutions leading to loss of catalytic activity. We here confirm that the HvGBSSIa enzyme from one such waxy barley variety, CDC_Aloamo, has a 90% reduction in catalytic activity. We also engineered plants with expression of transgenic C-terminal green fluorescent protein-tagged HvGBSSIa of both the non-waxy type and of the CDC_Aloamo type to monitor their subcellular localization patterns in grain endosperm. HvGBSSIa from non-waxy cultivars was found to localize in discrete concentric spheres strictly within starch granules. In contrast, HvGBSSIa from waxy CDC_Aloamo showed deficient starch targeting mostly into unknown subcellular bodies of 0.5–3 µm in size, indicating that the waxy phenotype of CDC_Aloamo is associated with deficient targeting of HvGBSSIa into starch granules.

Key words: Amylose, GBSS, starch biosynthesis, starch functionality, subcellular targeting, waxy.

Introduction

Starch is the main polysaccharide in which carbon and energy are stored in higher plants and is the principal dietary source of energy for humans (Zeeman et al., 2010; Tetlow, 2011). Cereal starch accounts for over 90% of the world market for starch (Tetlow, 2011) and is used in several industrial processes including papermaking and first generation bioethanol. Starch is an insoluble, semi-crystalline glucan composed of two polymers of glucose—amylose and amylopectin—that are linked together by α-1,4-D-glycosidic linkages with branch points formed by α-1,6-D-glycosidic linkages. The highly branched amylopectin is the major component of starch and has an estimated molecular mass of...
10⁷–10⁸ Da whereas the essentially linear amylose is smaller than amylopectin (10³–10⁶ Da) (Buléon et al., 1998). The ratio between amylose and amylopectin in plant starches varies depending on the botanical source, but typically 15–30% of the starch granule is composed of amylose (Tetlow, 2011). This ratio seems to have been optimized in nature for optimal starch granule robustness, digestibility and carbohydrate remobilization as demonstrated for barley seedling establishment (Shaik et al., 2014). Waxy starches are characterized by either being amylese-free (waxy) or having low amylose content (near-waxy) (<5%) (Domon et al., 2002). The texture of cooked starches is influenced by the content of amylose, and the clear starch pastes with good freeze–thaw stability and little tendency to retrograde that are formed by waxy starches are of high value in many processed foods (Denyer et al., 2001). Also, the cloning and characterization of starch modifying enzymes is increasingly important for industrial and agro-biotechnological modification of starches and other carbohydrates (Hebelstrup et al., 2015).

Starch synthesis in cereals involves five classes of enzymes: ADP-glucose pyrophosphorylase (AGPase), starch synthases (ADP-α-D-glucose-[1→4]-α-D-glucan 4-α-D-glucosyltransferase; EC 2.4.1.21, GT5), starch phosphorylase, and starch branching and debranching enzymes (Blennow et al., 2013). Five classes of starch synthases have been identified in barley: granule-bound starch synthase (GBSSI) and starch synthases SSII, SSIII, and SSIV. All five classes catalyse the transfer of a glucosyl unit from ADP-glucose to the non-reducing end of an existing α-glucan chain (Zeeman et al., 2010). Pioneering work in the 1960s suggested that amylose synthesis is strictly associated with activity of granule-bound starch synthase(s) (reviewed by Nelson and Pan, 1995). Mutations in GBSSIa genes have later been verified in many waxy mutants of cereal crops, where in particular waxy corn (Wessler and Varagona, 1985; Nelson and Pan, 1995) and glutinous rice (Wang et al., 1995; Olsen and Purugganan, 2002) are the major waxy crops produced. Of the two GBSS genes in barley, only HvGBSSIa is expressed in the endosperm (Radchuk et al., 2009), whereas HvGBSS Ib is expressed in the pericarp and the embryo (Sreenivasulu et al., 2006). GBSS-type enzymes represent the majority of the protein fraction bound within storage starch granules (Wang et al., 2013), but they do not contain known types of starch binding domains. HvGBSSIa is targeted to amyloplasts by the recognition of an N-terminal transit peptide, which is cleaved off during translocation across plastid membranes. We have previously shown that the first 69 N-terminal amino acids of HvGBSSI a corresponds to such a transit peptide, which can effectively target transgenic enhanced green fluorescent protein (eGFP) to the amyloplast of barley endosperm cells (Hebelstrup et al., 2010). In Arabidopsis, targeting of GBSS to transient leaf starch granules has been shown to be dependent on interaction with PTST (protein targeting to starch), which is a 26 kDa protein containing a glucan-binding family 48 carbohydrate-binding module (CBM48) (Seung et al., 2015). In barley, storage starch accumulates in the starch endosperm starting around 6 days after pollination and continues throughout the grain-filling period (Radchuk et al., 2009). During grain development the amylose content increases from around 20% to around 30% in the mature grain (Radchuk et al., 2009). The loss of activity from GBBS Ia in barley was linked to the waxy phenotype by Hylton et al. (1996) and several waxy barley cultivars have since then been identified and characterized (Domon et al., 2002; Pat ron et al., 2002; Ma et al., 2010; Asare et al., 2012). Most waxy barley mutants are a result of either low or no expression of the GBSSIa gene caused by deletions or non-silent single nucleotide polymorphisms (SNPs) (Domon et al., 2002; Patron et al., 2002). However, there are some waxy cultivars with no detectable amylose wherein the full GBSSIa gene is expressed at a normal level, and therefore non-silent SNPs in the GBSSIa gene are interpreted to result in catalytic inactivity without affecting enzyme concentration (Asare et al., 2012). However, the enzymes of these presumed loss-of-function alleles were never cloned and characterized in vitro. CDC Alamo is an example of such a waxy barley cultivar, where HvGBSSIa is expressed at a normal level (Pat ron et al., 2002; Asare et al., 2012). Here we describe what effect the SNPs identified in the HvGBSSIa gene of the waxy barley cultivar CDC Alamo have on the catalytic properties of HvGBSSIa and its subcellular localization. We found that HvGBSSIa from non-waxy cultivars are effectively targeted into concentric spheres within developing starch granules. In contrast, HvGBSSIa from the waxy cultivar CDC Alamo has a 90% loss of catalytic activity, and it is not correctly localized in starch granules. Instead it is localized mostly into unknown subcellular bodies with a size of 0.5–3 μm.

**Material and methods**

**Constructs for expression of barley granule-bound starch synthases Ia**

Barley granule-bound starch synthase (HvGBSSIa, GenBank accession: AAM74048) was codon optimized for expression in *E. coli* and synthesized by DNA2.0 (www.DNA20.com). It was synthesized without the initial 69 amino acid residues, i.e. plastid transit peptide as predicted using TargetP (http://www.cbs.dtu.dk/services/TargetP/). The HvGBSSIa gene contained additional NdeI and HindIII restriction sites at the 5’ and 3’ end, respectively, to facilitate re-cloning into the pET28a expression vector, which contains a 6×His tag. For expression in barley plants we used the construct pUCE_Ubi:HvGBSSIa-eGFP: NOS, which we have described previously (Carciofi et al., 2012). In brief, this construct was made by cloning the original wild-type full length HvGBSSIa cDNA sequence (including the transit peptide) to generate the plasmid p241-HvG- BSSIa-eGFP (full length HvGBSSIa with a C-terminally linked green fluorescent protein (GFP) tag). Full length HvGBSSIa-eGFP was then re-cloned into the pUCE plant transformation vector Ubi:USER:NOS (Hebelstrup et al., 2010).

**Site-directed mutagenesis**

Mutants for kinetic and in planta studies were constructed by using the QuickChange site-directed mutagenesis kit (Agilent Technologies) with pET28a-HvGBSSIa and pJ241-HvGBSSIa-eGFP as templates (respectively for expression either in *E. coli* or in barley), and primers listed in Supplementary Table S1 at JXB online. Sequence-confirmed mutants of HvGBSSIa-eGFP were digested with PstI (Fermentas, FastDigest) and re-cloned into the
Expression and purification of barley starch synthases

The expression vectors of HvGBSSIa or mutants were transformed into E. coli Tuner(DE3) cells, which were grown in 1 liter LB medium containing 50 µg ml⁻¹ kanamycin to OD ~0.6, cooled on ice for 10 min, induced with 50 µM isopropyl β-D-1-thiogalactopyranoside and incubated overnight at 16 °C. The cell pellet was resuspended in 10 ml buffer A (20 mM Tris–HCl, pH 8.0, 500 mM NaCl, 40 mM imidazole, 1 M UREA, 10% (v/v) glycerol) per gram pellet together with two protease inhibitor tablets (Roche 11 873580001) and two to three drops of antifoam (Sigma-Aldrich). The cell suspension was lysed using a continuous cell disruptor (1.35 kbar, Constant Systems Ltd). DNAseI (2 µg ml⁻¹, bovine pancreas, grade II, Roche 101041559001) and MgCl₂ (final concentration 10 mM) were added to the cell lysate and incubated on ice for 20 min before centrifugation (Beckman, JA-20, 48 400 g, 30 min, 4 °C). The filtered supernatant was loaded on a 6 ml HisTrap column (GE Healthcare), equilibrated with buffer A and eluted using a gradient from 0% to 45% buffer B (as buffer A but with 500 mM imidazole). The eluted fractions were mixed with DTT and EDTA, pH 8.0 (final concentration 10 mM of both) and analysed using SDS-PAGE. Fractions containing HvGBSS were pooled, concentrated using a Vivaspin20 centrifugal concentrator (30 kDa molecular mass cut-off) and loaded on a Superdex75 gel-filtration column (GE Healthcare), equilibrated with buffer C (20 mM Tris–HCl, pH 8.0, 150 mM NaCl, 10% (v/v) glycerol, 1 mM DTT, 1 mM EDTA). Fractions were analysed using SDS-PAGE and those containing HvGBSSIa were pooled. The protein concentration was estimated using the Bradford method with bovine immunoglobulin as a reference. Enzyme concentrations for activity assays were estimated as acceptor. Reactions were initiated by addition of 1 mM ADP-glucose, di-ammonium salt was prepared using chemo-enzymatic synthesis as described previously (Cuesta-Seijo et al., 2013). Initial rates were determined by coupling the release of ADP to NADH oxidation via pyruvate kinase and lactate dehydrogenase in a protocol adapted from (Gosselin et al., 1994). Assays were performed in a final volume of 100 µl with the following final concentrations: 50 mM Bicine, pH 8.5, 25 mM potassium acetate, 0.1% (w/v) bovine serum albumin, 2 mM MgCl₂, 10 mM DTT, 0.375 mM NADH, 0.7 mM phosphoenolpyruvate tricarboxylylaminon salt, 6 U ml⁻¹ pyruvate kinase, and 30 U ml⁻¹ lactate dehydrogenase (both Sigma-Aldrich, rabbit muscle type II) with 30–800 nM enzyme at 37 °C; 10 mM maltotriose (Sigma-Aldrich M8378) or 1 mg ml⁻¹ of glycogen (Sigma-Aldrich G8876, rabbit liver type III) was used as acceptor. Reactions were initiated by addition of 1 mM ADP-glucose. Enzyme concentrations for activity assays were estimated by the method of Bradford with bovine immunoglobulin as a reference. NADH oxidation was monitored by the decrease in absorbance at 340 nm. For single substrate kinetics, the fixed substrate concentration used was >5 times the Kₘ value. Kₘ and Vₘ values were calculated by fitting the Michaelis–Menten equation to the initial rates (GraphPad Prism version 4.03, GraphPad Software, San Diego, CA, USA).

Western blotting

Developing endosperm was ground in buffer (10 mM Tris–HCl, pH 8.0) in an automatic tissue lyser (FastPrep™ FP120, Thermo Savant) for 20 s. The samples were spun twice at 200 g for 2 min. The supernatant was collected, and it was checked with a microscope that it did not contain any starch granules. The pellet, which consisted of starch granules, was washed 10 times with buffer. Protein concentrations were determined with Bradford reagent (Bio-Rad no. 500-0006) using BSA as standard. The samples were mixed with a 6× SDS-loading buffer to reach the following final concentrations: 50 mM Tris–HCl, pH 6.8, 10% (v/v) glycerol, 1% SDS, 3% β-mercaptoethanol. The samples were boiled for 10 min and the proteins were separated by SDS-PAGE using NuPAGE Novex Bis-Tris Mini Gels (4–12%, Invitrogen) and Bio-Rad MiniProtein II Multiscreen Apparatus according to the manufacturer’s instructions (Bio-Rad bulletin 1721). Proteins were transferred to polyvinylidene fluoride membranes. Rabbit antiserum raised against HvGBSSIa (Carlsberg Laboratory, Copenhagen, Denmark) was used to detect HvGBSSIa at a dilution of (1:1000). Purified antibodies from rabbit serum raised against eGFP (Sigma-Aldrich, G1544) at a dilution of (1:1000) was used to detect eGFP-tagged HvGBSSIa. The secondary antibody (1:5000) was goat anti-rabbit purified IgG coupled with alkaline phosphatase (Sigma-Aldrich, A3687).

Crossing and genotyping

Transgenic plants with overexpression of the GBSSIa variants were grown in a controlled growth chamber (12–14 °C) under a 16 h day (550 µmol m⁻² s⁻¹) with a 10–95% relative humidity. Spikes without non-mature green anthers were identified and emasculated 2 days before pollination with mature anthers from the three varieties: CDC Alamo, CDC Candle and SB94912 of H. vulgare. F₁ grains were harvested at maturity and a new generation was propagated to obtain mature F₂ grains. Embryos were removed from the F₁ grains for genomic DNA purification using the FastDNA® Kit (MP Biomedicals, LLC, OH, USA). The F₂ grains were genotyped with respect to the GBSSIa gene by PCR as described by (Asare et al., 2012). In brief, the primers Hor_wx_1F (5’-AACAAAAAGCGAAGGAAAGA-3’) and Hor_wx_1R (5’-AGAATCGAACCAACCGAGGT-3’) were used to identify the GBSSIa genotypes of CDC Candle and SB94912, and the primers Hor_wx_5F (5’-TTTGTCATGGTCCTCC-3’) and Hor_wx_5R (5’-TCCGATCTAATGATGATC-3’) were used to identify the CDC Alamo GBSSIa genotype (Asare et al., 2012).

Epifluorescence and confocal laser scanning microscopy

Endosperm cells and starch granules from T₁ and F₂ grains were analysed by fluorescence, Normarski/differential interference contrast (DIC) and transmission light microscopy on a Zeiss Axioplan 2 epifluorescence microscope equipped with an AxioCam MRc5. The filter B1001 (supplied by the manufacturer) was used for detection of eGFP fluorescence and the filter B1000 was used for detection of endosperm cell wall autofluorescence. For transmission light microscopy, starch granules were stained by Lugol’s iodine stain solution (250 mg I₂, 2.5 g KI, 125 mL ddH₂O) (Carciofi et al., 2012) to identify the waxy phenotype. Granular localization of GFP-tagged GBSSIa and mutants was also determined using a Leica TCS SP5-X
MP UV confocal microscope equipped with filters for eGFP or chlorophyll detection. Endosperm was isolated from developing grains harvested 30 days after pollination and from dry mature grains or mature grains imbibed in sterile ddH₂O for 2 h. Relative fluorescence from starch granules was determined by using the software supplied by the manufacturer. No fluorescence was detected in starch granules from non-transformed barley grains or in grains from vector control (pUCE_Uhc_eGFP) transformants.

Amylose determination and qPCR
The amylose content of barley grains was determined using iodine colorimetry as described previously (Wickramasinghe et al., 2009) and expression levels of HvGBSSIa mRNA was determined by qPCR as described previously (Carciofi et al., 2012).

Results
Enzymatic activity of HvGBSSIa from CDC Alamo
A single SNP which causes an Asp219→Val219 (D219V) mutation in the HvGBSSIa allele of CDC Alamo (HvGBSSIaCDC_Alamo) has previously been reported (Asare et al., 2012). However, aligning the cDNA sequence of the HvGBSSIaCDC_Alamo allele with a number of other HvGBSSIa alleles shows that there are also two other SNPs in HvGBSSIaCDC_Alamo, which deviate from the consensus sequence of HvGBSSIa (Supplementary Table S2). They cause a Met490→Val490 (M490V) mutation and an Ile491→Val491 (I491V) mutation, respectively (Fig. 1A). The structure of GBSSIa from rice has been determined (Momma and Fujimoto, 2012). We mapped D219V, M490V, and I491V to this structure (Fig. 1B). However, none of these amino acids is located in the presumed catalytic site or in the transit peptide. To test if they are important for the efficiency of GBSSIa starch synthesis, we cloned and successfully expressed six variants (HvGBSSIaCDC_Alamo) containing all three amino acid substitutions D219V/M490V/I491V, and four different combinations of these, namely (i) HvGBSSIaM490V, (ii) HvGBSSIaI491V, (iii) HvGBSSIaM490V/I491V, (iv) HvGBSSIaD219V, (v) HvGBSSIaM490V/I491V, and (vi) HvGBSSIaD219V. High enzyme yields in E. coli (>50 mg l⁻¹) were achieved for all six variants. To test the efficiency of each variant, substrate kinetics were determined for all six with either rabbit liver glycogen or ADP-Glc as varying substrate. All five tested variants (HvGBSSIaD219V, HvGBSSIaM490V, HvGBSSIaI491V, HvGBSSIaM490V/I491V, and HvGBSSIaCDC_Alamo) showed activity in vitro, and hence none of the amino acid substitutions resulted in a full loss of catalytic activity (Table 1). The strongest reduction in catalytic activity was observed for HvGBSSIaCDC_Alamo which showed a 90% reduction of $k_{cat}$ compared with HvGBSSIaWT.

Starch targeting patterns of HvGBSSIa
HvGBSSIaCDC_Alamo had a strong reduction in catalytic activity, but was not fully inactive. To examine the subcellular localization patterns of GBSSIa, we generated transgenic barley plants (var: Golden Promise, GP) from calli transformed with constructs for overexpression of either HvGBSSIaCDC_Alamo or HvGBSSIaWT as fusion proteins tagged with eGFP. GBSS-type enzymes are strictly associated with starch granules. In our previous work, we found that HvGBSSIaWT-eGFP fusion protein expressed in callus cells was strictly localized to starch granules (Carciofi et al., 2012), whereas such visual localization of GBSS within storage starch granules from tubers or grains has not previously been reported. Barley grain starch has a bimodal granule morphology consisting of large disc-shaped granules of 15–50 μm (A-type) and small granules of 2–3 μm (B-type) (Jane et al., 1994; Carciofi et al., 2012). Barley callus cell starch granules are much smaller (<3 μm) than the large A-type granules from grains (Carciofi et al., 2012). We therefore studied the cellular localization pattern of HvGBSSIaCDC_Alamo-eGFP and HvGBSSIaWT-eGFP in endosperm cells in grains from two independent transgenic barley lines for each of the HvGBSSIa varieties. Starch granules were detected with

![Fig. 1](image-url)
transmitted light using DIC settings. HvGBSSI<sub>a</sub><sup>CDC_Candle</sup>-eGFP and HvGBSSI<sub>a</sub><sup>WT</sup>-eGFP were detected as green epifluorescence. Cell walls were detected as blue fluorescence. In endosperm cells of developing grains (30 days after pollination (DAP)), HvGBSSI<sub>a</sub><sup>WT</sup>-eGFP was strictly associated with the starch granules (Fig. 2A–C), whereas HvGBSSI<sub>a</sub><sup>CDC_Candle</sup>-eGFP showed a deficient starch targeting pattern with localization into smaller subcellular bodies rather than into starch granules (Fig. 2D–F). Western blotting with antiserum against HvGBSSI on either the protein fraction of the supernatant or on starch granules purified from developing endosperm showed bands with similar size in the supernatant and starch granules from CDC Candle or starch granules of GP. These bands correspond well to the expected size (59 kDa) of HvGBSSI<sub>a</sub> without a transit peptide. While GBSSI<sub>a</sub> was strictly associated with starch granules in GP, GBSSI<sub>a</sub> in CDC_Candle was detected mainly in the supernatant, with only a smaller quantity in the starch granules (Fig. 2G). Western blotting with eGFP antibody similarly confirmed that transgenic HvGBSSI<sub>a</sub><sup>WT</sup>-eGFP was located in purified starch granules, whereas HvGBSSI<sub>a</sub><sup>CDC_Candle</sup>-eGFP was mostly associated with the supernatant, rather than starch granules (Supplementary Fig. S1). The localizations, as detected by the eGFP signal of HvGBSSI<sub>a</sub><sup>WT</sup>-eGFP and HvGBSSI<sub>a</sub><sup>CDC_Candle</sup>-eGFP into starch granules and the smaller subcellular bodies, were further investigated by confocal laser scanning microscopy (Fig. 3). This showed that for both HvGBSSI<sub>a</sub><sup>WT</sup>-eGFP and HvGBSSI<sub>a</sub><sup>CDC_Candle</sup>-eGFP fluorescence was detected in both large (A-type) and small (B-type) starch granules. However, HvGBSSI<sub>a</sub><sup>WT</sup>-eGFP (Fig. 3A) was more strongly associated with starch granules than HvGBSSI<sub>a</sub><sup>CDC_Candle</sup>-eGFP (Fig. 3B) when comparing relative difference in granule fluorescence (Fig. 3C). The subcellular bodies associated with HvGBSSI<sub>a</sub><sup>CDC_Candle</sup>-eGFP were mostly disc shaped with less fluorescence in the center and a diameter of 0.5–3 µm (Fig. 3D). They disappeared completely after a 10 min treatment with protease K, leaving both large (A-type) and small (B-type) starch granules intact (Fig. 3E). This suggests that they may be protein bodies. Interestingly, both HvGBSSI<sub>a</sub>-eGFP types were not uniformly distributed within the starch granules but were concentrated in internal concentric spheres (Fig. 3F–G). These spheres became blurred after a 15 min treatment with α-amylase (Fig. 3H; only HvGBSSI<sub>a</sub><sup>WT</sup> is shown, although HvGBSSI<sub>a</sub><sup>CDC_Candle</sup> showed a similar blurring pattern). We recorded both transmitted light (with DIC) and eGFP to simultaneously visualize endosperm subcellular structures and HvGBSSI<sub>a</sub><sup>CDC_Candle</sup>-eGFP subcellular bodies in thin (50 µm) sections of developing endosperm (Fig. 3I, J). While some of the subcellular bodies seemed to be located away from starch granules (Fig. 3J), we were unable to determine if the subcellular bodies were located inside or outside of the amyloplast membrane. However, in leaves where HvGBSSI<sub>a</sub><sup>CDC_Candle</sup>-eGFP and HvGBSSI<sub>a</sub><sup>WT</sup>-eGFP were also expressed, we observed that HvGBSSI<sub>a</sub><sup>WT</sup>-eGFP was localized inside chloroplasts (Fig. 3K), whereas HvGBSSI<sub>a</sub><sup>CDC_Candle</sup>-eGFP was mostly located outside of chloroplasts (Fig. 3L). In dry, post-harvest mature grains there was only a slight tendency for distribution into concentric spheres in both HvGBSSI<sub>a</sub><sup>WT</sup>-eGFP and HvGBSSI<sub>a</sub><sup>CDC_Candle</sup>-eGFP starch granules (Fig. 4A, B) and the difference in relative levels of fluorescence between HvGBSSI<sub>a</sub><sup>CDC_Candle</sup>-eGFP and HvGBSSI<sub>a</sub><sup>WT</sup>-eGFP starch granules was smaller (Fig. 4C) than during endosperm development (Fig. 3C).

Deficient starch targeting of HvGBSSI<sub>a</sub><sup>CDC_Candle</sup>-eGFP is associated with the waxy phenotype

Golden Promise is a non-waxy variety with fairly high amylose content, and overexpression of either HvGBSSI<sub>a</sub><sup>WT</sup>-eGFP or HvGBSSI<sub>a</sub><sup>CDC_Candle</sup>-eGFP in Golden Promise did not change the amylose content, suggesting that GBSSI<sub>a</sub> enzyme activity may already be saturated in this variety (not shown). Therefore, to further demonstrate the link between the deficient GBSSI<sub>a</sub><sup>CDC_Candle</sup>-eGFP starch targeting pattern with the waxy phenotype of CDC_Candle, we crossed the transgenic barley plants expressing either GBSSI<sub>a</sub><sup>CDC_Candle</sup>-eGFP or HvGBSSI<sub>a</sub><sup>WT</sup>-eGFP with either CDC_Candle or either of the near-waxy barley varieties CDC Candle or SB94912 (♀ Golden Promise_Ubi:GBSSI<sub>a</sub><sup>WT</sup> or CDC_Candle)<sub>a</sub> × σ CDC_Candle or CDC_Candle × SB94912). Both of the HvGBSSI<sub>a</sub><sup>CDC_Candle</sup> and HvGBSSI<sub>a</sub><sup>SB94912</sup> alleles contain a deletion in the promoter and have low expression levels of HvGBSSI<sub>a</sub> enzyme imparting a near-waxy
starch phenotype (Asare et al., 2012). The near-waxy starch phenotype can be identified by a screen with iodine staining of endosperm flour, which will reveal near-waxy starch granules staining with a red color, in contrast to non-waxy starch granules, which are blue. Parents and F2 grains of the crosses were genotyped with respect to the endogenous HvGBSSIa gene as described in 'Materials and methods' to identify F2 grains that are homozygous for one of the endogenous waxy alleles HvGBSSIa\textsuperscript{CDC Alamo}, HvGBSSIa\textsuperscript{CDC Candle} or HvGBSSIa\textsuperscript{SB94912}. Expression of HvGBSSIa\textsuperscript{CDC Alamo}-eGFP and HvGBSSIa\textsuperscript{WT}-eGFP in the endosperm cells of F1 and segregating F2 grains was detectable with fluorescence microscopy as described above for T1 grains, and also showed localization patterns identical to those observed in T1 grains: HvGBSSIa\textsuperscript{WT}-eGFP was strictly targeted to starch granules (Fig. 5A, E), whereas HvGBSSIa\textsuperscript{CDC Alamo}-eGFP showed a deficient starch targeting pattern with localization into subcellular bodies rather than into starch granules (Fig. 5C, G). Furthermore, there was a difference between HvGBSSIa\textsuperscript{CDC Alamo}-eGFP and HvGBSSIa\textsuperscript{WT}-eGFP in their ability to rescue the waxy phenotype of CDC Alamo, CDC Candle or SB94912: transgenic expression of HvGBSSIa\textsuperscript{WT}-eGFP rescued the three waxy varieties by making non-waxy blue-staining granules (Fig. 5B, F) with an amylose content above 30% in mature grains (Fig. 5J), whereas the three waxy lines with over-expression of HvGBSSIa\textsuperscript{CDC Alamo}-eGFP retained red-staining waxy starch granules with an amylose content below detection level (Fig. 5J). The effect was the same in the three different cultivars, showing that the deficient starch targeting pattern of HvGBSSIa\textsuperscript{CDC Alamo}-eGFP as well as the inability of HvGBSSIa\textsuperscript{CDC Alamo}-eGFP to rescue the waxy phenotype is independent of both the endogenous HvGBSSIa locus and the genetic background in the three different waxy varieties used in this study. The results for SB94912 were identical to those of CDC Candle. So for simplicity, only CDC Candle is shown in Fig. 5. To ensure that the observed deficiencies of HvGBSSIa\textsuperscript{CDC Alamo}-eGFP was not due to a difference in expression efficiency between the HvGBSSIa\textsuperscript{CDC Alamo}-eGFP and HvGBSSIa\textsuperscript{WT}-eGFP transgene, mRNA expression levels of total HvGBSSIa were

![Image](image-url)
measured by qPCR, which demonstrated that expression of \( \text{HvGBSSI}_{\text{WT}} \)-eGFP was not significantly lower than expression of \( \text{HvGBSSI}_{\text{CDC-Alamo}} \)-eGFP. Two independent lines of \( \text{HvGBSSI}_{\text{CDC-Alamo}} \)-eGFP expression were used (lines 5 and 12) and both failed to complement \( \text{waxy} \) in any of the three cultivars.

**Discussion**

The amylose-free phenotype of barley cv. CDC Alamo has been presumed to be the result of a complete elimination of HvGBSSI activity caused by a single SNP alteration from adenine to thymine, which replaces the aspartic acid with the
hydrophobic amino acid valine at position 219 (Patron et al., 2002; Asare et al., 2012). This mutation was never tested in vitro and only measurement of starch synthase activity associated with granules in developing endosperm of barley cv. CDC Alamo suggested that most or all of GBSSIa protein was inactive (Patron et al., 2002). In addition, a re-examination of the gene entry of barley cv. CDC Alamo (AF486519) revealed two extra SNPs that also resulted in two mutations, M490V and I491V that may have some importance for protein structure and function. Asp219 (HvGBSS1 numbering) is also in close proximity with the basic Arg196 and Lys229 residues, which could potentially be disturbed by the Asp219 itself is conserved among different plant GBSSI genes (Leterrier et al., 2008). However, Asp219 itself is conserved among different plant GBSSI genes (Leterrier et al., 2008), indicating that it may have some importance for protein structure and function. Asp219 (HvGBSS1 numbering) is also in close proximity with the basic Arg196 and Lys229 residues, which could potentially be disturbed by the Asp219→Val substitution in HvGBSS1CDC_AlamoeGFP (A) and HvGBSS1A WT-eGFP (B). (C) Relative fluorescence intensities in HvGBSS1A WT-eGFP and HvGBSS1CDC_AlamoeGFP starch granules (n=10) from two different lines each: HvGBSS1CDC_AlamoeGFP (lines 12 and 5) and HvGBSS1A WT-eGFP (lines 1 and 2). Bars indicate standard errors.

Examination of Asp219, Met490, and Ile491 in a modeled structure based on the published structure of rice GBSS1a, which shares very close homology with barley HvGBSS1a (Momma and Fujimoto, 2012), did not provide an immediate explanation as to why HvGBSS in CDC Alamo should be inactive (Fig. 1B). GBSS1a belongs to the GT5 family of glycosyltransferases in the CAZy database (Coutinho et al., 2003), which adopts the characteristic GT-B fold (double Rossmann fold). The interface between the two Rossmann folds forms the catalytic site wherein an ADP molecule was identified in the rice GBSS1a structure (Momma and Fujimoto, 2012). Asp219 is neither positioned in this catalytic site nor involved in any putative substrate binding, but is located in the 380s loop of starch synthases; a region that is highly variable among all starch synthase genes (Leterrier et al., 2008). However, Asp219 itself is conserved among different plant GBSSI genes (Leterrier et al., 2008), indicating that it may have some importance for protein structure and function. Asp219 (HvGBSS1 numbering) is also in close proximity with the basic Arg196 and Lys229 residues, which could potentially be disturbed by the Asp219→Val substitution in HvGBSS1CDC_AlamoeGFP (A) and HvGBSS1A WT-eGFP (B). (C) Relative fluorescence intensities in HvGBSS1A WT-eGFP and HvGBSS1CDC_AlamoeGFP starch granules (n=10) from two different lines each: HvGBSS1CDC_AlamoeGFP (lines 12 and 5) and HvGBSS1A WT-eGFP (lines 1 and 2). Bars indicate standard errors.

The three identified substitutions D219V, M490V, and I491V were selected as targets for a mutational study of HvGBSS1a to explain the amylose-free phenotype of CDC Alamo. Western blotting with HvGBSS1a antiserum showed only a small reduction in activity (44–59%) compared with wild-type (HvGBSS1aWT), while HvGBSS1aM490V/I491V (9–47%) and HvGBSS1aCDC_Alamoe (6–30%) activities were much lower. The catalytic properties of HvGBSS1a1491V were essentially unchanged compared with wild-type; however, HvGBSS1aM490V differed significantly from the wild-type by having at least three-fold higher $k_{cat}$. The $K_{m}$ value towards ADP-glucose HvGBSS1a was in the same range as those previously determined for maize GBSS (Macdonald and Preiss, 1985), but notably lower than that of potato (Edwards et al., 1999) and Chlamydomonas reinhardtii GBSS (Delrue et al., 1992) (10- and 23-fold lower, respectively). In summary, the combination of the three SNPs in the HvGBSS1a gene of CDC Alamo seems to lead to strong reduction in catalytic activity of the enzyme.

Our results indicate that the waxy phenotype is also associated with deficient targeting of HvGBSS1a into starch granules in CDC Alamo. We used eGFP tagging to demonstrate that a consensus HvGBSS1a from non-waxy cultivars is targeted to starch granules, where it is localized into concentric spheres. This is well in line with the general consensus that GBSS-type enzymes are bound tightly to starch granules within the internal granular matrix (Tatge et al., 1999; Wang et al., 2013). Concentric spheres of similar morphology were observed in wheat, maize, and potato starch in a confocal laser scanning microscopy study of starch treated with a protein-specific dye (3-(4-carboxybenzoyl) quinoline-2-carboxaldehyde) (Han and Hamaker, 2002). In line with our observations, these spheres are likely to be GBSSIa, as the concentric spheres were not observed in waxy maize and amylose-free potato starch, which correlated with the lack of expression of GBSSIa (Han and Hamaker, 2002).

In contrast, the targeting of HvGBSS1a is deficient in CDC Alamo. Western blotting with HvGBSS1a antiserum showed that HvGBSS1aCDC_Alamoe fails to be fully targeted to starch granules in CDC Alamo, where a major fraction of HvGBSS1aCDC_Alamoe is found in the soluble fraction of developing endosperms (Fig. 2G). Transgenic expression of eGFP-tagged HvGBSS1aWT or HvGBSS1aCDC_Alamoe confirmed that HvGBSS1aCDC_Alamoe is unable to target correctly to starch granules and to synthesize amylose in vivo (Fig. 5). HvGBSS1a is generally targeted to amyloplasts by a transit peptide that is cleaved when the protein is transported through the amyloplast membrane. Our previous work has demonstrated that a transit peptide, with an identical 69 amino-acid sequence to that of HvGBSS1aWT and HvGBSS1aCDC_Alamoe (which have identical gene sequences for the transit peptide), effectively

Fig. 4. (A, B) Confocal laser scanning microscopy of starch granules from mature dry grains of transgenic barley plants expressing HvGBSS1aCDC_Alamoe-eGFP (A) and HvGBSS1aWT-eGFP (B). (C) Relative fluorescence intensities in HvGBSS1aWT-eGFP and HvGBSS1aCDC_Alamoe-eGFP starch granules (n=10) from two different lines each: HvGBSS1aCDC_Alamoe-eGFP (lines 12 and 5) and HvGBSS1aWT-eGFP (lines 1 and 2). Bars indicate standard errors.
transports transgenic eGFP to the amyloplast of developing barley endosperm cells (Hebelstrup et al., 2010). This suggests that the incorrect targeting of HvGBSSIa\textsuperscript{CDC_Alamo} is not due to a deficient transit peptide. This is supported by the western blot (Fig. 2G), which shows that the soluble fraction of HvGBSSIa\textsuperscript{CDC_Alamo} in CDC Alamo has an identical size to that of the HvGBSSIa\textsuperscript{CDC_Alamo} that is correctly targeted to starch granules. This implies that the transit peptide must have been correctly removed from the soluble fraction of HvGBSSIa\textsuperscript{CDC_Alamo}.

GBSSI seems to be the predominant protein in storage starch granules (Wang et al., 2013). In contrast, soluble starch synthases and starch branching enzymes form complexes (Tetlow et al., 2008; Liu et al., 2012). However, GBSS-type enzymes do not contain any identified starch binding modules, nor do they interact in the mentioned complexes with the soluble starch synthases or starch branching enzymes containing starch binding modules. In Arabidopsis leaves, amylose synthesis is dependent on a GBSS type that is targeted to transient starch granules by interaction with the protein PTST (protein targeting to starch), which contains a carbohydrate binding module, and loss-of-function \textit{ptst} mutant Arabidopsis plants synthesize a waxy-like type of transient starch in their leaves (Seung et al., 2015). A PTST gene homolog exists in the barley genome, and therefore the same mechanism may be necessary for correct targeting of HvGBSSIa to storage starch granules and synthesis of amylose in barley grain endosperm. This also demonstrates that mutations in other genes than GBSS can contribute to the waxy phenotype. However, in CDC Alamo, the three identified mutations in HvGBSSIa seem to be the single cause of deficient targeting to starch granules and the waxy phenotype, because HvGBSSIa\textsuperscript{CDC_Alamo} was also incorrectly targeted and unable to rescue the waxy phenotype when expressed in the cultivars CDC Candle and SB94912 (Fig. 5).
which are waxy due to a deletion in the gene promoter and subsequently lack HvGBSSIa enzyme (Asare et al., 2012). Similarly, the waxy phenotype could be rescued in CDC Alamo by expression of functional HvGBSSIaWT resulting in >30% amylopectin content. Given that binding of GBSS with PTST-type proteins is necessary for correct targeting of GBSS and synthesis of amylose (Seung et al., 2015), it is possible that one or more of the three amino acid substitution (D219V, M490V, and I491V) in HvGBSSIaCDC-Alamo in barley prevents binding to a putative PTST protein, whereby HvGBSSIaCDC-Alamo is incorrectly targeted. However, the binding of GBSS to PTST in Arabidopsis was mediated through a coiled-coil domain identified on the distal helix (Fig. 5B), which is not in the structural area of the three amino acid substitutions of HvGBSSIaCDC-Alamo.

We cannot exclude the possibility that the deficient targeting of HvGBSSIa in CDC Alamo is caused indirectly by the partial loss of catalytic activity. It can also be speculated that HvGBSSIa is a less processive variant, which causes it to fail to remain bound to (or within) the starch granules after corpectargeting. The cellular structure of the subcellular bodies where HvGBSSIaCDC-Alamo-eGFP is located is not clear (Figs 2, 3 and 5). Their sensitivity to proteinase K indicates that they are protein-rich bodies. In leaves, at least some of them are located outside of chloroplasts (Fig. 3L). In endosperm cells, it was not clear if they are located outside or on the inside of the amyloplast membrane. However, as discussed above, western blotting suggests that their transit peptide has been correctly removed, indicating that they have been translocated across a plastid membrane. It cannot be excluded that they represent more than one type of cellular structure. They do not seem to be breakdown products of HvGBSSIa, since the western blot showed a single intact protein in the soluble fraction of CDC Alamo with a size similar to that of the HvGBSSIa in the starch granules in Golden Promise (Fig. 2G). Similar results were obtained for a transgene system where HvGBSSIaCDC-Alamo-eGFP is expressed in endosperm located mostly to the supernetant with a similar size to that of the correctly targeted HvGBSSIaWT-eGFP and neither showed signs of breakdown products (Supplementary Fig. S1). In summary, our work suggests that the waxy phenotype of CDC Alamo is associated with a combination of a strong reduction of catalytic activity of HvGBSSIa, as well as a deficiency of either targeting or retaining HvGBSSIa within the matrix of starch granules.

Supplementary data

Supplemental data are available at JXB online.

Fig. S1. Western blot of transgenic lines.

Table S1. Primers.

Table S2. Alignment of selected barley GBSSI sequences from various normal and waxy cultivars.

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