Expression of starch-binding factor CBM20 in barley plastids controls the number of starch granules and the level of CO₂ fixation

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Abstract

The biosynthesis of starch granules in plant plastids is coordinated by the orchestrated action of transferases, hydro-lases, and dinkinases. These enzymes either contain starch-binding domain(s) themselves, or are dependent on direct interactions with co-factors containing starch-binding domains. As a means to competitively interfere with existing starch–protein interactions, we expressed the protein module Carbohydrate-Binding Motif 20 (CBM20), which has a very high affinity for starch, ectopically in barley plastids. This interference resulted in an increase in the number of starch granules in chloroplasts and in formation of compound starch granules in grain amyloplasts, which is unusual for barley. More importantly, we observed a photosystem-independent inhibition of CO₂ fixation, with a subsequent reduced growth rate and lower accumulation of carbohydrates with effects throughout the metabolome, including lower accumulation of transient leaf starch. Our results demonstrate the importance of endogenous starch–protein interactions for controlling starch granule morphology and number, and plant growth, as substantiated by a metabolic link between starch–protein interactions and control of CO₂ fixation in chloroplasts.

Keywords: Barley, Hordeum vulgare, photosynthesis, starch metabolism, starch-protein interactions.

Introduction

Biosynthesis of starch granules in plant plastids is carried out by the orchestrated action of enzymes directly involved in, or related to, α-glucan elongation and modification. Elongation is carried out by specialized glycosyltransferases termed starch synthases (SSs), which use ADP-glucose as donors of glucose units. Subsequently, α-1,6 branches are introduced by starch
branching enzymes (SBEs). Maltooligosaccharides (MOSs) may act as primers for starch granule initiation and synthesis. MOSs are synthesized from units of glucose-1-phosphate by a combined action of α-glucan phosphorylase and α-1,6 starch branching enzymes (for reviews see Blennow et al., 2013; Tetlow and Emes, 2017). Starch granules are between 1–100 µm in size, with different morphologies depending on the species, such as round, ovoid, or polyhedral (Bertoft, 2017). In extreme cases, such as in starch granules that consist only of amylose, more amorphous granules are formed (Carciofi et al., 2012a). Regular starch granules have so-called growth rings with a thickness of 0.2–0.5 µm that alternate between semi-crystalline and amorphous blocklets (Goldstein et al., 2017).

Little is known about the organization of enzymes within and on the surface of the starch granules during their synthesis. Some starch-related enzymes bind through starch-binding domains (Pfister and Zeeman, 2016); however, others, such as granule-bound starch synthases (GBSSs), do not have such domains. Instead, these enzymes may interact with co-factors with starch-binding domains that guide them to starch granules. GBSSs have been shown to be transported to the granules by Protein Targeting to Starch 1 (PTST1) (Seung et al., 2015). The GBSS is then trapped within the granules during their synthesis and is clearly associated with the growth rings of the granules (Hebelstrup et al., 2017). In line with this, the proteome of starch granules to a large extent consists of GBSSs (Cao et al., 2015). Starch synthases operate by enzymatic mechanisms that are either processive or distributive. In a processive mechanism, elongation continues at the same acceptor α-glucan chain once it has initiated. In a distributive mechanism, the elongation is terminated after a limited number of reactions, and then it re-initiates at a new acceptor chain. Soluble starch synthases are generally not completely trapped within the starch granule, possibly because they elongate starch chains by a purely distributive mechanism (Cuesta-Seijo et al., 2015). In contrast, GBSSs operate by a processive mechanism, and hence they feature more largely in the proteome of starch granules than the soluble starch synthases (Cao et al., 2015). Previous studies have shown that loss of processive ability of GBSS results in it being trapped in small structures (≤1 µm) that are distributed separately from the starch granules (Hebelstrup et al., 2017). This indicates that the processive mechanism of GBSS is the reason that it is granule-bound. Plant starches mostly serve two different functions. Transient starch in chloroplasts is a way to temporarily store for later use carbohydrate that is formed during periods of high photosynthesis. In the leaf mesophyll this can usually be detected as a diurnal cycle of starch content, with transient reserves peaking at the end of the day and becoming minimal at the end of the night. In contrast, storage starches are deposited mainly for reproductive purposes in amyleplasts for a much longer time, often in seeds and storage organs such as tubers. Due to their different physiological functions, there are significant differences in biosynthesis and structure between transient and storage starches. In addition, granules of storage starches vary between organs and species, and between cultivars of individual crops. For example, rice and oat grain starch granules are of a compound type, where several small granules combine into larger ones, which often break apart into smaller polyhedral and sharp-edged granules during purification of granules in agricultural processing. It has been suggested that this characteristic shape is determined within amyleplasts by septum-like structures that separate individual granules (Yun and Kawagoe, 2010; Kawagoe, 2013). For transient leaf starch, there seems to be a link between chloroplast division and the number of granules per chloroplast (Crumpton-Taylor et al., 2012).

The mechanisms for granule initiation in both storage and transient starches are mostly unknown. There are several mutants with decreased numbers of storage granules per chloroplast, suggesting that the mutated proteins are involved in starch initiation. Mutation of the gene for soluble starch synthase IV (SSIV) in Arabidopsis results in the number of starch granules per chloroplast being limited to one. Although SSIV is a starch-interacting protein, it does not contain any known starch-binding domain. Instead, it binds though coiled-coil domains to the co-factor Protein Targeting to Starch 2 (PTST2), which has a starch-binding domain of the Carbohydrate-Binding Motif 48 (CBM48) family, and it has been suggested that PTST2 mediates the interaction between SSIV and MOSs for the initiation of transient leaf starch granules in Arabidopsis (Seung et al., 2017). PTST2 also binds to other plastidial proteins such as the thylakoid-associated protein MFP1, which is involved in granule initiation (Seung et al., 2018). Arabidopsis does not make storage starch, but a homolog of PTST2 in rice called FLO6 has been characterized that seems to be involved in the formation of grain storage granules, since their compound-like pattern is partly lost in flo6 loss-of-function mutants (Peng et al., 2014). A homolog of PTST2/FLO6 called FRA exists in barley, and fra loss-of-function mutants have fractured starch granules (Saito et al., 2018). The interactions of carbohydrate-binding domains with starch have also been shown to form a structural basis for control of the catalytic activity of enzymes in signal transduction. An example of this is the regulatory protein SnRK1 that is involved in responses to low-energy stress and in sensing of cellular carbon status. Subunits of SnRK1 contain carbohydrate-binding motifs that regulate its activity negatively when bound to starch (Ávila-Castañeda et al., 2014) and positively when bound to maltose (Ruiz-Gayoso et al., 2018).

Despite of the obvious link between photosynthesis and starch biosynthesis, surprisingly few studies have examined starch metabolism in relation to control of photosynthesis. Photosynthesis is divided in two linked mechanisms: the light reactions that take place in chloroplast thylakoid membranes, where PSI and PSII harvest photons to produce NADPH and ATP; and the carbon reactions where NADPH and ATP are consumed by stromal enzymes of the Calvin–Benson cycle to fix CO₂. ssIV mutants and in particular ssIV ssIV double-mutants show signs of inhibition of both the carbon reactions, as indicated by reduced CO₂ assimilation, and the light reactions, as indicated by reduced PSII efficiency. This inhibition of photosynthesis seems to be due to an accumulation of ADP–glucose, the content of which is more than 100-fold higher in these mutants than in wild-type plants (Ragel et al., 2013). These mutants also accumulate significantly higher levels of sucrose, which down-regulate photosynthesis.
There are different families of carbohydrate-binding motifs (CBMs). Some starch synthases and starch-modifying enzymes contain CBMs that allow them to target to starch granules (Pfister and Zeeman, 2016) whilst others depend on indirect interactions through other proteins that contain CBMs (Seung et al., 2015; Pfister and Zeeman, 2016). Glucoamylase from Aspergillus niger contains a domain of the CBM20 family that has a very high affinity for starch (Christiansen et al., 2009; Cuesta-Seijo et al., 2015; Tanackovic et al., 2016), and in this present study we examined whether this module (hereafter termed simply CBM20) may provide a means by which to explore starch–protein interactions in plastids. Our assumption was that since CBM20 has a very strong affinity for starch, it will competitively interfere with the large array of starch–protein interactions in plastids. We constructed seven independent transgenic lines of barley that ectopically express a synthetic protein, TP-CBM20-eGFP, in plastids. This protein consists of a transit peptide from barley Granule-Bound Starch Synthase Iα (GBSSIα), the CBM20 domain, and the fluorescent tag eGFP. The transit peptide guides the protein to plastids. It does not have any catalytic domains but despite this, the CBM20-expressing plants showed an increased number of disorganized starch granules in the chloroplasts. The plants also showed a transition to compound-type storage granules in grains, a significantly decreased C:N ratio in the leaves, and significantly lower photosynthesis, which seemed to be specifically regulated at the level of the Calvin–Benson cycle.

Materials and methods

Engineering of vectors and transformation of barley

Independent transgenic lines of barley (Hordeum vulgare cv Golden Promise) with expression of CBM20 from Aspergillus niger isolate G1 and vector control lines were generated by transformation with Agrobacterium tumefaciens (AGL0) (Hebelstrup et al., 2010; Carciofi et al., 2011, 2012b). The plasmid vectors pUCe-UBI:TP-eGFP:NOS and pUCE-UBI:TP-eGFP-CBM20:NOS were generated by In-Fusion® HD cloning (Takara Bio Europe) into the vector pUCE-UBI:TP:NOS (Hebelstrup et al., 2010), which has a unique PacI restriction site (TTAATTTA) between the transit peptide (TP) and the termination site (NOS). Primers used to clone the green fluorescent protein (eGFP) were: forward, 5′-CGCGCTGAGGTGGCCACTGGAGGCAGGAG-3′ and reverse, 5′-GATCAGACTGGATTATTAAATGAGCCGGAAGGCTTATGTTGATGATGAGCTTGTACAG-3′.

GFP was added in the plasmid as a marker to visualize the transgenic tissue in the embryonic callus. CBM20 was synthesized artificially by GenScript, and the codon was optimized for expression in H. vulgare. The primers used were: forward, 5′-CTGCAGAGGCGCTTATGTTGATGATGAGCTTGTACAG-3′ and reverse, 5′-GATCAGACTGGATTATTAAATGAGCCGGAAGGCTTATGTTGATGATGAGCTTGTACAG-3′. The primers used for the cloning procedure were designed following the In-Fusion protocols.

Plants were grown in a growth chambers with a 16/8 h light/dark photoperiod and temperature 12–14 °C.

Western blotting and measurements of Rubisco content

The 2nd leaves from the top of the plants (3.5 months old) were collected from the transgenic lines TP-eGFP-CBM20 and TP-eGFP and from the Golden Promise wild-type (WT). Leaf samples (100 mg FW) were ground in an automatic cell homogenizer (FastPrep™ FP120, Thermo Savant) for 20 s. Extraction buffer [1.0 ml 50 mmol l−1 Tris, 5 mmol l−1 β-mercaptoethanol, 12.5 % (v/v) glycerol] was added to ground samples and mixed by vortexing. The mixture was then centrifuged at 15 000 g for 15 min at 4 °C. The pellet was mixed with 100 μl 1× SDS-loading buffer [10 mmol l−1 Tris, 1% (w/v) SDS, 10 % (v/v) glycerol, 0.4 mg ml−1 bromophenol blue] and the samples were boiled for 10 min and centrifuged at 15 000 g for 2 min. The supernatant was used for SDS-PAGE with NuPAGE Novex (ThermoFisher Scientific).

Bis-Tris Mini Gels (4–12%, Invitrogen) were used in conjunction with Bio–Rad Mini-ProTEAN II Multiscreen Apparatus according to the manufacturer’s instructions (Bio–Rad bulletin 1721). Purified antibodies from rabbit serum raised against eGFP (Sigma-Aldrich, G1544) at a dilution of (1:1000) were used to detect eGFP-tagged TP or TP-CBM20. The secondary antibody (1:5000) was goat anti-rabbit purified IgG coupled with alkaline phosphatase (Sigma-Aldrich, A3687).

Quantification of Rubisco from SDS–PAGE gels was conducted using Image Lab software. During the quantification procedure, a standard protein BSA (0.1 mg ml−1) was separately loaded in 5, 10, 15, and 20 μl volumes to four lanes on the same gel. The standard proteins were used to give a standard curve of known concentration. The content of Rubisco in each sample lane was then quantified.

Confocal laser-scanning microscopy

Granular localization in leaves was observed using an Olympus Fluoview FV1000 confocal microscope as described previously (Carciofi et al., 2012b; Hebelstrup et al., 2017). No fluorescence was detected in starch granules from non-transformed WT grains.

Endosperm was isolated from dry mature grains of transgenic plant TP-eGFP-CBM20 and WT plants. Transmission confocal laser-scanning microscopy was used to examine the morphology of starch granules.

Primary metabolite analysis

Relative levels of primary metabolites were measured as described in Hasler-Sheetal et al. (2016). In brief, 50-mg FW samples (2nd leaves from 4-month-old plants) from six independent T0 CBM20-expressing plants and six vector control plants were extracted for 2 min in an ultrasound bath followed by 15 min on a thermo-shaker (both at 4°C) in 1 ml methanol/acetonitrile/water (4:4:2 v/v/v) chilled to −20°C and spiked with 0.4 mg l−1 13C6 sorbitol and reserpine as internal standards. After centrifugation at 16 000 g for 5 min, the relative metabolite levels were analysed by GC–quadrupole time-of-flight MS (7200 GC-QTOF MS, Agilent Technologies).

Staining of starch in leaves and purification of leaf starch

The second leaves from 3.5-month-old vector control plants and four independent T0 TP-eGFP-CBM20 plants were collected after a 12-h light period or a 24-h dark period. Pigments were extracted in 80% (v/v) ethanol at 80 °C for 30 min and then stained for 10 min with Lugol’s iodine solution (stock: 250 mg I2, 2.5 g KI, 125 ml ddH2O; freshly diluted 10 000 x in 100 mM HCl). The Lugol solution was removed after ~10 min for 2 min. The supernatant was used for SDS-PAGE.

Photosynthetic fluorescence induction curves

To evaluate differences in PSII efficiency between vector control and T0 TP-eGFP-CBM20 plants, fluorescence induction curves were determined. Plants (three replicates) were dark-adapted for 40 min at room temperature. Measurements were taken with a MINI-PAM chlorophyll
fluorescence meter (Waltz). Prior to dark adaptation, the leaf-clip holder 2030-B fitted with the standard MINI-PAM/F fiber optics (both Waltz) was attached to the latest fully unfolded leaf. The MINI-PAM set points followed factory settings except for actinic light, which was set to 19 μmol m⁻² s⁻¹. F-zero was set once prior to measurements. The measuring light was turned on 30 s before starting a measurement. Measurements were carried out over a period of 5 h that was approximately centred around the middle of the photoperiod.

**CO₂ response curves and photosynthetic light-response curves**

Mean CO₂ response (A/C) curves for three WT and three independent T₀ CBM20-expressing plants were obtained using a LI6400 (LI-COR) coupled to a 6400-02 red LED light source. The automated A/C program for the LI6400 was utilized with the following settings: leaf area, 3 cm²; bulk temperature, 20 °C; light, 1500 μmol m⁻² s⁻¹; and CO₂ concentrations of 400, 300, 250, 200, 150, 100, 50, 40, 30, 20, 10, 0, 1000, 1200, 1500, and 1800 ppm. One measurement was taken per CO₂ level on the 2nd leaf from the top of the plant. Plants were 4 months old. Prior to starting, the leaf was kept in the leaf chamber for 20 min. The intervals between readings were 10–12 min, except that an interval of 20 mins was used after increasing the CO₂ from 50 ppm up to 400 ppm. Measurements were done at room temperature on the latest fully unfolded leaf. The leaf vapor deficit varied between 0.7–2.0 kPa. Photosynthetic light-response curves (A/Q) were also determined. The basic settings were the same as for the A/C curves with the exception that the CO₂ level was constant at 400 ppm, and the values of photosynthetically active radiation (PAR) in the chamber were 400, 350, 250, 150, 120, 60, 40, 30, 20, 10, 0, 1000, 1500, and 1800 μmol m⁻² s⁻¹. Modelling of the A/C curves was done using ‘fitaci’ from the R package plantecophys (Duursma, 2015). Prior to modelling of the A/C curves, all start measurements at 400 ppm CO₂ were removed and the model parameters were kept as standard. Modelling focused on the maximum rate of Rubisco carboxylation (c,max), the CO₂ compensation point (C_i), and the photosynthetic rate of CO₂ fixation (A_i). The model was also fitted with the response curve was solved as the full model with four parameters (A_i, α, A_m, Θ), the Thornthwaite model, with the exception that the CO₂ level was constant (Tanackovic et al., 2016). We added an N-terminal transit peptide (TP) from barley granule-bound starch synthase (GBSS1a) to the modeling. The triose phosphate use (TPU) part of the model for T_ype-I errors by using the multivariate techniques available in R.

Where A_i is the net rate of photosynthesis in light (μmol CO₂ m⁻² s⁻¹), A_m is the maximum capacity of photosynthesis at 400 ppm, α is the apparent quantum yield (mol mol⁻¹), I is the irradiance in the PAR region (μmol m⁻² s⁻¹), Θ is the convexity (dimensionless), and Rₖ,A/Q is the respiration in the light (μmol CO₂ m⁻² s⁻¹).

First, the mathematical non-rectangular hyperbola used for the A/Q response curve was solved as the full model with four parameters (A_m, α, Θ, and Rₖ,A/Q). As Θ depends on several factors (Ogren and Evans, 1993) interpretation is limited, and hence the A/Q models were re-calculated with Θ fixed at 0.85, Akaike information criteria values (AIC) on the Θ parameter showed in general no restrictions for this reduction of the mathematical non-rectangular hyperbola.

**C/N ratio**

The 2nd leaf from the top of 4-month-old plants were sampled from WT, vector control, and independent T₀ CBM20-expressing lines (six plants each). All samples were freeze-dried for 48 h and ground through a 1-mm sieve. Nitrogen and carbon concentrations in the ground samples were measured using elemental analyser (vario EL-III, Germany).

**Results**

**Expression of CBM20 in plastids**

We generated plants with ectopic expression of the starch-binding domain (CBM20) from glucoamylase of A. niger G1 (1,4-α-D-glucan glucohydrolase, E.C. 3.2.1.3). This variant of CBM20 has a very strong affinity for starch and α-glucan (Tanackovic et al., 2016). We added an N-terminal transit peptide (TP) from barley granule-bound starch synthase (GBSS1a) as previous studies have shown that this TP effectively targets the transgenic proteins to plastids, and that it is effectively cleaved off during passage of the protein over the plastid envelope membranes (Carciófi et al., 2012a; Hebelstrup et al., 2017). To visualize the location of the transgenic CBM20 by fluorescence microscopy, we added the fluorescent tag eGFP to the construct, generating the TP-eGFP-CBM20 protein. For the vector control, we also generated plants with only the TP and the eGFP domains (TP-eGFP). The TP (67 aa) has a molecular weight of 7 kDa, eGFP (238 aa) has a molecular weight of 27 kDa, and CBM20 (108 aa) has a molecular weight of 12 kDa. Therefore, if the pre-protein of this construct is translocated into the plastids and the N-terminal TP is cleaved off, the final molecular weight of eGFP-CBM20 would be 39 kDa. Using western blotting with antiserum raised against eGFP, we confirmed the presence of a band at that size in extracts from leaves of transgenic (T₀) plants expressing the TP-eGFP-CBM20 construct (Fig. 1A). There was no sign of the non-cleaved pre-protein (46 kDa). Similarly, for the vector control expressing TP-eGFP, a band with the expected size of eGFP (27 kDa) was observed, and there were no signs of a non-cleaved protein (34 kDa). Fluorescence microscopy confirmed that the transgenic TP-eGFP-CBM20 as well as the vector control TP-eGFP were correctly targeted to chloroplasts in leaves (Fig. 2).

We produced seven independent lines of T₀ plants with expression of TP-eGFP-CBM20. Six of these were used in the subsequent analysis. They all showed very slow growth and after 4 months the TP-eGFP vector controls and the WT plants were much larger than those expressing CBM20 in plastids (Fig. 1B). CBM20-expressing plants continued to produce new tillers that could be propagated vegetatively; however, the stem apical meristem of the tillers either remained dormant or the stem grew only a few centimeters above the root (not shown). Only on one occasion did a single tiller eventually develop into a spike, which produced only one seed. One of the independent T₀ plants with only low expression of the transgenic CBM20 (Fig. 1A, lane 4) showed less retardation of growth than lines with higher levels of expression (lane 3). Several of the vector control lines showed levels of transgenic eGFP that were higher than those of the eGFP-CBM20 lines (Fig. 1A, lane 2), but none of them showed any sign of growth retardation, excluding a growth effect of eGFP itself.

**Starch granule numbers in chloroplasts**

We used confocal laser scanning microscopy to observe the localization of eGFP in the chloroplasts of the seven T₀ lines.
and the vector controls (Fig. 2A–F). Chloroplasts were identified by red autofluorescence from chlorophyll (Fig. 2B, E, H). Vector controls showed a distribution of eGFP throughout the chloroplast, in agreement with a stromal localization (Fig. 2F), whereas eGFP-CBM20 was localized in small granules, in agreement with its expected binding to starch (Fig. 2C, Supplementary Fig. S2 at JXB online). These granules disappeared when leaves were kept in darkness for 24 h.
Surprisingly, there were many more and smaller starch granules in the eGFP-CBM20 plants than in the vector controls, and they often contained only one or two granules per chloroplast (Fig. 3, Supplementary Fig. S3). In addition, the granules in eGFP-CBM20 plants were randomly located, whereas those in the vector controls were well organized toward the center of the chloroplasts. This was evident both by fluorescence detection (Fig. 2, Supplementary Figs S2, S3) and by iodine staining (Fig. 3). To further examine the binding of eGFP-CBM20, we purified leaf starch granules to confirm their eGFP fluorescence (Fig. 3C). A similar eGFP signal was not detected in granules from the vector controls (Fig. 3E, F). The results of SDS-PAGE of proteins from the leaf starch granules were in agreement with the presence of the 39-kD eGFP-CBM20 protein (Supplementary Fig. S4), and demonstrated a difference in the ratio of individual levels of endogenous starch-bound proteins between the transgenic and vector control plants.

**Starch levels and metabolism**

To determine how the expression of CBM20 affected metabolism, we measured the relative concentrations of primary metabolites in leaves from six independent T0 plants at mid-day using GC-MS analysis. We focused on primary metabolites that are part of the Calvin–Benson cycle, glycolysis, and the citric acid cycle and on amino acid metabolism related to these pathways (Fig. 4). For the Calvin–Benson cycle, we identified relative concentrations of the intermediates 3-phosphoglyceric acid (PGA) and glyceraldehyde-3-phosphate (G3P). PGA is the product of the first step of the fixation in the cycle, where RuBP reacts with CO2 to form two molecules of PGA in a reaction catalysed by Rubisco. G3P is the output metabolite of the cycle and in plastids it can be converted into ADP-glucose, which enters the starch biosynthesis pathway. Alternatively, when the leaves act as a source tissue, G3P is exported from the plastid to the cytosol where it is converted into sucrose, which is the major metabolite of the water-soluble carbohydrate pool in plant leaves. G3P exported from the plastid may also enter the glycolytic pathway in the cytosol. The concentrations of PGA, G3P, and sucrose as well as fructose and glucose were all significantly lower in eGFP-CBM20-expressing leaves than in vector control leaves, and the content of Rubisco was also lower Supplementary Fig. S1. This indicated lower photosynthetic productivity in plants with expression of eGFP-CBM20.

When glucose and other carbohydrates are metabolized in leaves they enter the glycolytic pathway. We identified and quantified the levels of the final products of cytosolic glycolysis, namely phosphoenolpyruvate (PEP) and pyruvate. In accordance with lower levels of the sugars that are the input for
glycolysis, the levels of PEP and pyruvate were also lower. The relative concentrations of metabolites in the citric acid cycle were generally lower in plants expressing eGFP-CBM20 compared with the vector controls except for oxaloacetate, which is a substrate of citrate synthase that also uses acetyl-CoA produced from pyruvate. This step is the metabolic link between glycolysis and the citric acid cycle, and the accumulation of oxaloacetate may therefore have been due to lack of pyruvate. To examine whether the low levels of photoassimilates had an impact on starch biosynthesis, we produced a qualitative score of relative starch levels in leaves at the end of periods of light and darkness by means of iodine staining (Fig. 5). Starch levels were generally highest near the central leaf veins, and were higher in leaves from vector control plants than in those containing CBM20-bound starch, both at end of periods of light and darkness. Interestingly, the contents of glutamine and glutamate, both with a high N/C ratio, were higher in eGFP-CBM20 plants than in vector controls (Fig. 4). This was consistent with weaker carbon fixation relative to N assimilation in eGFP-CBM20 plants, which was confirmed by elemental analysis that showed a higher N content and lower carbon to nitrogen (C:N) ratio in these plants (Fig. 6).

Photosynthesis

The relatively lower level of the products of photosynthesis in the plants with expression of eGFP-CBM20 in the chloroplasts suggested that they might have a lower level of CO₂ fixation, and so we produced A/Cᵢ and A/Q curves. The value of Rₐ/Aₒ did not vary significantly between vector control and CBM20-expressing plants (Table 1). Significant differences were observed for Vₖₐₓ and Jₖₐₓ according to the A/Cᵢ curves (Fig. 7A–C), which suggested a lower level of CO₂ fixation in the eGFP-CBM20 plants. Stomatal densities and aperatures were also found to be significantly different (Table 1). Using the data from the A/Q curves, stomatal conductance (gₛ) was evaluated under ambient conditions (CO₂ 400 ppm; PAR 1500 μmol m⁻² s⁻¹). No differences in gₛ were observed between vector control and eGFP-CBM20 plants, whilst Cᵢ values were significant higher in the eGFP-CBM20 plants (Table 1). The difference in Cᵢ seemed to originate from lower CO₂ accumulation as evidenced by the reduced photosynthetic rate (Aₛ) in the eGFP-CBM20 plants. That Aₛ was the limiting factor was further confirmed by the fact that the relationship between photosynthetic rate and stomatal conductance was more dependent on Aₛ than gₛ (Fig. 7C). Differences between eGFP-CBM20 and vector control plants were found for both the slope (P≤0.001) and the intercept (P≤0.01).

The value of Fᵥ/Fₘ was slightly but significantly, lower in eGFP-CBM20 plants than in vector controls (Table 1). However, as the contents of Rubisco and the RuBP product 3-PGA were lower in eGFP-CBM20, a photosynthetic protection mechanism might have been activated continuously and this could have been reflected in the difference in Fᵥ/Fₘ. To investigate this, we evaluated the parameters quantum yield of PSII (Yₚₛ), quantum yield of light-induced non-photochemical quenching (Yₚₒ), and quantum yield of non-regulated heat dissipation and fluorescence emission (Yₜₒ). Yₚₛ was lower for
Starch–protein interactions in barley plastids

CBM20-expressing plants compared to the vector controls (Fig. 7D). Excess energy was dispersed through $Y_{NPQ}$, a photo-protective mechanism, as pH- and/or xanthophyll-regulated thermal dissipation (Fig. 7E). In addition, excess energy was also dispersed through passive dissipation of energy in the form of heat and fluorescence, mainly due to the closed PSII reaction centers ($Y_{NO}$) (Fig. 7F). At the end of the dark adaptation, a steep increase in $Y_{NPQ}$ indicated that CBM20 activated photo-protective mechanisms that removed energy by heat loss, which was seen as a peak in $Y_{NPQ}$ within the first 80 s. Within this period, the $Y_{NO}$ in CBM20-expressing plants decreased rapidly to a level comparable with the vector control, indicating opening of PSII reaction centers that had previously closed been closed by the first saturated pulse ($F_{m}$). After 145s, $Y_{NPQ}$ in CBM20-expressing plants reached the level of the vector control whereas $Y_{NO}$ decreased more slowly than in the vector control, being significantly higher between 167–294 s.

Starch granule formation in amyloplasts

We examined the morphology of starch grains from mature endosperm by transmission light confocal laser-scanning microscopy. Starch granules in barley endosperm are normally divided in two populations according to size, namely A-granules that have diameters in the range 8–15 µm and B-granules in the range 1–3 µm. Whilst the granules in the vector control plants had the normal appearance of large A- and small B-granules, CBM20-expressing plants had a compound structure, with
Table 1. Photosynthesis and stomatal parameters for barley plants expressing TP-eGFP-CBM20 and vector control plants

| Parameter                          | Vector control | CBM20  | t-test |
|------------------------------------|----------------|--------|--------|
| A/C                                |                |        |        |
| V_{\text{Cmax}} (\mu mol CO_2 \text{m}^{-2} \text{s}^{-1}) | 101.2 ± 7.7    | 34.5 ± 1.8 | **    |
| J_{\text{max}} (\mu mol e^{-} \text{m}^{-2} \text{s}^{-1}) | 122.8 ± 4.0    | 58.9 ± 8.1 | **    |
| R_{\text{L/AO}} (\mu mol CO_2 \text{m}^{-2} \text{s}^{-1}) | 0.50 ± 0.65    | 0.62 ± 0.22 | NS    |
| A/Q                                |                |        |        |
| α (mol mol^{-1})                   | 0.02 ± 0.002   | 0.03 ± 0.006 | NS    |
| A_{\text{a}} (\mu mol CO_2 \text{m}^{-2} \text{s}^{-1}) | 15.7 ± 1.0     | 8.1 ± 1.0 | **    |
| R_{\text{L/AO}} (\mu mol CO_2 \text{m}^{-2} \text{s}^{-1}) | 0.25 ± 0.08    | 0.03 ± 0.37 | NS    |
| Photosynthesis at 400 μmol CO_2 mol^{-1} and PAR 1500 μmol m^{-2} s^{-1} | |        |        |
| g_{\text{i}} (mol H_2O mol^{-1})   | 0.20 ± 0.02    | 0.21 ± 0.03 | NS    |
| A_{\text{i}} (μmol CO_2 m^{-2} s^{-1}) | 14.7 ± 1.1     | 8.0 ± 0.9 | **    |
| C_{\text{i}} (μmol CO_2 mol^{-1})  | 256.0 ± 5.3    | 322.3 ± 1.5 | ***    |
| F_{\text{i}}/F_{\text{m}}        | 0.79 ± 0.004   | 0.77 ± 0.004 | *     |
| Stomata                            |                |        |        |
| Length (μm)                        | 31.33 ± 0.71   | 29.34 ± 0.78 | *     |
| Height (μm)                        | 17.61 ± 0.40   | 15.51 ± 0.41 | ***    |
| Aperture (μm)                      | 6.94 ± 0.33    | 3.84 ± 0.44 | ***    |
| Density (number mm^{-2})           | 91.7 ± 1.1     | 143.4 ± 3.1 | ***    |

A/C; PAR was set to 1500 μmol m^{-2} s^{-1}. V_{\text{Cmax}}, maximum velocity of Rubisco carboxylation; J_{\text{max}}, maximum rate of electron transport demand for RUBP regeneration; R_{\text{L/AO}}, A/C; curve estimation of respiration rate in the light.

Discussion

CBM20 binds to starch granules in transgenic plants

Starch synthases and other starch-related enzymes interact with starch during its synthesis. Some do this directly through carbohydrate-binding modules (CBMs) of the starch-binding domain type (SBDs) whereas others interact with CBM-containing co-factors. CBMs are classified into different families, as presented in the Carbohydrate-Active enZYmes database (www.cazy.org) (Lombard et al., 2014). At present, the database contains 85 different families, of which 13 are classified as SBDs. CBM20 is one of these SBDs, and entries with CBM20 modules are reported from organisms with broad phylogenetic diversity, including archaea, bacteria, and eukaryotes (Christiansen et al., 2009). The CBM20 used in the present study was the 12 kDa starch-binding domain (residues 509–616) of glycoamylase from Aspergillus niger, which was chosen based on a specific and high affinity for starch (Tanackovic et al., 2016). The affinity of CBM20 for starch is also significantly higher than that of the barley starch synthases GBSSI, SSI, SSIIa, SSIIIa, and SSIV (Cuesta-Seijo et al., 2015).

The starch synthases GBSSI, SSI, SSII, and SSIV do not have any known CBMs; however, SSIII contains a CBM25 (Pfister and Zeeman, 2016). Other factors involved in starch biosynthesis also have CBMs. Starch-branching enzymes, starch-debranching enzymes, and members of the PTST family have CBM48 domains. Starch granules are located in plastids, and therefore in order to target the CBM20 transgenes to these, we fused it with the transit peptide from GBSSI, which we have previously shown to be effective for protein translocation across the barley plastid envelope membrane (Hebelstrup et al., 2010, 2017). Western blotting of plants expressing either TP-eGFP (vector controls) or TP-CBM20-eGFP confirmed that the transgenic pre-proteins were correctly processed by cleaving off the 67-bp N-terminal TP (Fig. 1A). In agreement with this, confocal microscopy showed that both types of proteins were translocated across the chloroplast envelope in leaves and both were localized within the chloroplast (Fig. 2, Supplementary Fig. S2). In the vector controls, eGFP was localized throughout the chloroplast (Fig. 2D, F), whereas eGFP-CBM20 was located in 5–10 randomly distributed granules within each chloroplast (Fig. 2A, C). By tagging GBSSIa with eGFP, we have previously shown that chloroplasts contain 1–2 starch granules, which are placed roughly in the middle of each chloroplast (Hebelstrup et al., 2017; see Supplementary Fig. S3 for an example). Here, we also found one large starch granule in the middle of each chloroplast in the vector control plants (Fig. 3B). In contrast, plants transformed with eGFP-CBM20 had 5–10 smaller starch granules in each chloroplast (Fig. 3A).

Binding of CBM20 to starch granules down-regulates the Calvin–Benson cycle

Plants with expression of eGFP-CBM20 bound to starch granules in chloroplasts had a significantly reduced CO_2 fixation rate (Table 1, Fig. 7). They also had a slow growth rate, which was not observed in vector control plants with overexpression of eGFP at a level that was similar (or higher) than that of the eGFP-CBM20 expression lines (Fig. 1B). This showed that the effect on growth and photosynthesis was not due to expression of eGFP, nor to its presence in the chloroplasts or its transport across the chloroplast envelope. By staining in whole leaves, we determined that plants with CBM20 bound to granules contained less starch than vector control lines both at end of periods of light and darkness (Fig. 5). It is possible that starch biosynthesis was reduced in plants with expression...
Control E F

T6P and sucrose levels up-regulate photosynthesis (Oszvald et al., 2013). However, in the CBM20 plants, CO2 assimilation by photosynthesis as well as the level of 3-PGA, which is the direct product of CO2 fixation by Rubisco, were significantly lower despite low levels of sucrose and many other central metabolites. This indicated that the presence of the CBM20 bound to the starch granules in the chloroplast had an inhibiting effect on photosynthesis. Photosynthesis can also be regulated at the level of photosystem II, but the α-values for both TP-eGFP-CBM20 and the WT indicated that, under low-light conditions, the rate of electron transport was not limiting for CO2 fixation. This suggests that the inhibition may have been acting directly at the level of CO2 fixation in the Calvin–Benson cycle. Since CBM20 binds to the starch granules, it does not interact directly with Rubisco or other enzymes of the Calvin–Benson cycle and therefore its effect on photosynthesis must involve co-factors that link its inhibitory effect with the cycle. An alternative explanation is that the binding of CBM20 competitively releases unknown starch-binding factors with an inhibitory effect on CO2 assimilation. However, we cannot exclude the possibility that there were small amounts of CBM20 in the stroma that were below the detection limits of our methods, which these may have bound with soluble glucans or other carbohydrates, thus causing the observed metabolic effects.

These conclusions about the metabolic effects of CBM20 expression are summarized in Fig. 9. The non-spiking phenotype of the CBM20 plants may be an effect of the general low

![Fig. 7. Photosynthesis in barley plants expressing TP-eGFP-CBM20 and in vector control plants (Control).](Image 207x473 to 215x481)
metabolite status of these plants, since a lack of sucrose can prevent flowering via a number of different signaling pathways (Oszvald et al., 2018). Reduced photosynthesis in the CBM20 plants also resulted in a decreased C:N ratio (Fig. 6).

In line with this, we observed a higher concentration of several amino acids in the CBM20 plants, most notably the high N-containing glutamate (Fig. 4).

Binding of CBM20 to starch granules compromises their development

Expression of CBM20 increased the number of starch granules in the barley chloroplasts from 1–2 centrally localized granules into 5–10 smaller granules that were randomly distributed (Fig. 2). Similarly, expression of CBM20 turned some of the single, oval granules in embryonic callus into double-compound granule types, and the endosperm A-type granules into multiple compound granule types (Fig. 8). Expression of tandem starch-binding domains in potato tubers has been shown to have a similar effect of turning potato starch granules that would otherwise usually be ovoid or round into compound granules of a smaller, polyhedral shape (Nazarian-Firouzabadi et al., 2012). This was interpreted as an effect of the tandem SBDs, and a structural model was proposed where
these would link smaller, initial granules into the compound type. We observed a similar effect in barley (Fig. 8), but the CBM20 SBD was expressed as a single protein rather than in tandem. Therefore, the effect was not likely to that of linking smaller granules into large, compound ones by bringing them together, unless CBM20 acts similarly to a tandem dimer because it contains two glucan binding sites (Sorimachi et al., 1997). Transient expression of CBM20 with a fluorescent tag has shown that it binds to the surface of starch granules and is absent from the granule interior (B. Svensson, Technical University of Denmark, pers. comm.). In our transgenic barley leaves, the starch granules were too small to conclude whether the CBM20 was bound throughout the granules or solely on the surface (Figs 2, 3). However, the larger starch granules that developed in the embryonic callus did not have much CBM20 trapped within them, and it was bound at the surface with particular high concentrations at the interface between the sub-granules of a compound granule (Fig. 8C). We therefore suggest that the actions of CBM20 that we observed in relation to metabolism and starch granule development were caused simply by it binding at the surface of the granules. CBM20 acts competitively towards amylolytic enzymes whether these have a SBD or not (Southall et al., 1999). CBM20 expression in the barley chloroplasts and amyloplasts is therefore likely to have an effect on numerous starch-interacting factors in these compartments. The effect may be directly competitive, or there may also be an effect of CBM20 on structural disruption of the starch, which in turn may have an indirect effect on the interaction of the starch granule surface with the endogenous starch-interacting factors. Barley endosperm starch granules are not of the compound type, whereas rice endosperm granules are. Formation of compound starch granules in rice is coordinated by formation of membrane septa inside plastids via a mechanism that shares similarities with that of plastid division (Yun and Kawagoe, 2009, 2010). In particular, these septa seem to derive from the plastidinner-envelope membrane (Kawagoe, 2013). This process must require proteins that mediate binding between the starch granules and the membrane. These factors are currently unknown, but it has been suggested that the protein PDV2, which is located in the plastid outer envelope, is involved in this interaction (Yun and Kawagoe, 2010). Another study has shown that a thylakoid-associated protein is involved in starch-granule interactions (Seung et al., 2018). Such a model involving starch-bound factors and their binding to plastid membranes where starch granules are coordinated within plastids is in agreement with several of our observations, namely the loss of coordination of starch granule positioning within the chloroplast; their increase in number; and the change in the endosperm towards compound-type granules. All of these deficiencies are likely to be effects of competitive binding of CBM20 to the starch. This in turn affects the interaction between starch granules and any factors that may mediate interactions between starch granules and plastid membranes. Loss of membrane organization in maize has been shown to have a similar effect on endosperm granule morphology, with simple, single starch granules being turned into compound types (Myers et al., 2011).

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