Photoperiodic control of carbon distribution during the floral transition in *Arabidopsis thaliana*

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SINOPSIS (400 characters long)

The distribution of carbon resources from starch into soluble sugars is crucial to fuel the diverse physiological processes that take place during the floral transition. A multidisciplinary study supports the control of sugar mobilization in *Arabidopsis* during photoperiodic flowering through the regulation of *Granule Bound Starch Synthase* (*GBSS)* expression by the key photoperiodic regulator *CONSTANS*.

ABSTRACT

Flowering is a crucial process that demands substantial resources. Carbon metabolism must be coordinated with development through a fine-tuning control that optimises fitness for any physiological need and growth stage of the plant. However, how sugar allocation is controlled during the floral transition is unknown. Recently, the role of a *CONSTANS* (*CO*) ortholog (*CrCO*) in the control of the photoperiod response in the green alga *Chlamydomonas reinhardtii* and its influence over starch metabolism was demonstrated. In this work, the analysis of transitory starch accumulation and glycan composition during the floral transition in *Arabidopsis* shows that it is controlled by photoperiod. Employing a multidisciplinary approach, a role for CO in the control of the level and time of expression of the *Granule Bound Starch Synthase* (*GBSS*) gene is demonstrated. The first detailed characterization of a *GBSS* mutant involved in transitory starch synthesis and the analyses of its flowering time phenotype in relation with its altered capacity to synthesize amylose and to promote the modification of the plant cell free sugar content is also described. Photoperiod modification of starch homeostasis by CO may be crucial to increase the sugar mobilization demanded by the floral transition, contributing to our understanding of the flowering process.

INTRODUCTION

Plant life cycle is strongly influenced by environmental conditions, which affect the capacity to obtain energy for growth and development (Nicotra et al., 2010). The floral transition is a crucial developmental decision for a plant, because failing to produce a reproductive signal at the correct time of the year has a serious influence on its capacity to yield descendants and, for this reason, it is strictly regulated (Casal et al., 2004). Based on the analysis of flowering time mutants in *Arabidopsis thaliana*, a network of genes involved in the regulation of the floral transition was identified (Koornneef et al., 1991). These genes respond to different external stimuli generating inductive or inhibitory signal cascades whose equilibrium ultimately decides the reproductive fate of the plant (Fornara et al., 2010). In *Arabidopsis*, temperature, through the *FLOWERING LOCUS C* (*FLC*) pathway, and light signals, through the *CONSTANS* (*CO*) – *FLOWERING LOCUS T* (*FT*) module, are key external conditions that influence the flowering transition, although internal cues such as hormones or age also have a strong influence on flowering time (Amasino, 2010). However, the effect of growing under diverse trophic conditions on a plant developmental program is still poorly understood. Numerous studies on the effect of sugars in flowering time in different species have been reported (Bernier et al., 1993; Lebon et al., 2008) but their influence on the floral transition in *Arabidopsis* remains ambiguous, promoting flowering in some cases (Corbesier et al., 1998; Roldán et al., 1999), while acting, in other reports, as floral inhibitors (Ohto et al., 2001). Recently, roles for sucrose dependent kinases (Baena-González et al., 2007) or for trehalose-6-phosphate (T6P) (Gómez et al., 2010; Wahl et al., 2013) in vegetative growth and flowering have been suggested, while a role for *IDD8* gene in the activation of Sucrose Synthase and its involvement on photoperiodic flowering has also been described (Seo et al., 2011). It is surprising then that, having sugars so important a role in flowering time, there is still no indication on how this control is exerted and how these signals are integrated in the existing flowering pathways. Here, we show that the photoperiodic pathway is directly involved in the capacity of the plant to mobilize sugars from starch during the floral transition and that this effect influences its reproductive capacity.

Starch is the most important form of carbon reserve in plants. Starch granules contain branched amylopectin and linear (low branched) amylose (Streb and Zeeman, 2012). Two main types of starch can be distinguished according to their function: storage starch and transitory starch. Long-term storage starch is found in reserve organs such as

tubers, endosperm, embryos, or roots, while transitory starch is present in photosynthetically active tissues such as leaves. Transitory starch is synthesized during the day and degraded during the night to cover the carbon and energy requirements of the plant while storage starch shows a much more stable amount. The starch biosynthetic pathway has been extensively studied in diverse species, and considerable progress has been made towards understanding the role of each enzymatic step needed to build the final structure of the starch granule (Zeeman et al., 2010). Starch biosynthesis is performed by four sequential enzymatic reactions catalysed by ADP-glucose pyrophosphorylase, starch synthase, starch branching enzyme and starch debranching enzyme (Ball and Morell, 2003; Zeeman et al., 2010). Starch synthases (SS) transfer the glucosyl group of ADP-glucose, the product of ADP-glucose pyrophosphorylase, to the non-reducing ends of growing starch molecules by establishing new α(1-4) bonds. Multiple isoforms of SS have been described, which can be grouped in two classes: Soluble Starch Synthases (SSS) and Granule-Bound Starch Synthases (GBSS).

GBSS is exclusively located in starch granules (Sivak et al., 1993). It was originally identified in maize kernels as the product of the *waxy* gene and biochemical and genetic studies have shown that GBSS is responsible for the synthesis of the linear glycan (amylose) in starch (Nelson and Pan, 1995; Ball et al., 1998; Denyer et al. 2001). Genes encoding the orthologous protein have been isolated from many different plant species such as potato (van der Steege et al., 1992), pea (Dry et al., 1992), barley (Rohde et al., 1988), wheat (Clark et al., 1991), *Antirrhinum* (Mérida et al., 1999) and *Arabidopsis* (Tenorio et al., 2003). Although the analysis of mutants has demonstrated that *GBSS* is responsible for the synthesis of amylose in storage organs of diverse plants (Smith et al., 1997; Zeeman et al., 2010), no *GBSS* mutant involved in the accumulation of transitory starch has been thoroughly characterized to date. In fact, most studies concerning starch synthesis have been carried out on storage organs because of the social and economic importance of the long-term reserve form of starch and the relative availability of both enzymes and product. However, considerably less information is available about starch synthesis in other organs and tissues of the plant, although changes in the synthesis and mobilization of transitory starch affecting processes such as growth rate, flowering time, and seed filling have been reported before (Bernier et al., 1993; Schulze et al., 1994). In this sense, it has been proposed that mobilization of the starch stored in leaves and stems into sucrose provides one of the early signals for the induction of flowering (Bernier et al., 1993).

Analysis of *Arabidopsis* starch-less mutants has shown that synthesis of starch is necessary, not only to maintain normal growth rates under a natural day / night regime, but also to promote other developmental changes such as flowering or seed filling (Periappuram et al., 2000; Ventriglia et al., 2008). Transitory starch accumulations, as well as *GBSS* mRNA levels, are under circadian regulation both in higher plants and algae (Mérida et al., 1999; Mittag et al., 2005; Ral et al., 2006). *GBSS* expression in *Chlamydomonas reinhardtii* is also under photoperiodic control, so that *CrCO*, an ortholog of *A. thaliana CO*, directly influences *CrGBSS* transcript levels in the alga (Serrano et al., 2009). *CO* plays a central role in the photoperiodic control of the floral transition by long days in *Arabidopsis*, inducing the expression of the *FT* gene, whose product has a strong florigenic activity (Fornara et al., 2010). The photoperiodic regulation of carbon metabolism observed in *Chlamydomonas* (Serrano et al., 2009) suggests that the flowering inductive function of *CO* in plants may not be exclusively restricted to the regulation of *FT* expression, but that it may also have a role in controlling metabolic components that provide resources for the floral transition.

In this work we show that amylose constitutes a dynamic polymer within transitory starch whose turnover depends on the photoperiodic regulation of *GBSS*. In fact, *gbs* mutants display a small but consistent delay in flowering time exclusively in LD and co-expression analysis suggests that *GBSS* plays a crucial role in the connection of sugars and photoperiodic flowering. We also describe that the dynamic response of starch polymer to day-length is controlled by *CONSTANS* through the modification of *GBSS* gene expression during the floral transition. Hence, by altering *GBSS* expression, the photoperiod pathway coordinates the florigenic signal trough *FT* with the burst of sugars needed to drive the flowering process. This mechanism may reflect an evolutionarily conserved photoperiodic signalling in higher photosynthetic eukaryotes aimed at regulating sugar availability for important physiological and developmental processes such as the timing of reproduction (Valverde, 2011).

RESULTS

Starch and soluble sugar content are modified by the flowering stage of the plant.

In order to assess the effect of day length on the production of transitory starch, we measured starch accumulation in leaves of *Arabidopsis* Col-0 plants grown in 16 h light and 8 h darkness (long day, LD) and 8 h light, 16 h darkness (short day, SD) in 24 h courses. It was interesting to monitor if starch accumulation pattern (Lu et al., 2005; Gibon et al., 2009) was influenced by the floral stage of the plant, so plant starch amount was measured in *Arabidopsis* leaves every 4 h for 24 h in LD and SD, two days previous and two days after the appearance of the floral bud (Figure 1A). Leaf starch consistently reached higher levels in LD than in SD but an effect caused by the floral transition was only observed in LD. Before flowering (BF, close circles) starch accumulation reached higher levels (up to 1/3 more at ZT16) than after flowering (AF, open circles). This effect could be observed in both Col-0 and L*er* (Supplemental Figure 1) as well as in a number of other *Arabidopsis* accessions (Ruiz et al., manuscript in preparation). In SD, levels of starch were reduced both BF and AF and no significant changes due to the floral transition were detected.

As starch dynamics often define the levels of free sugars in plant cells (Singh and Juliano, 1977; Lu et al., 2005), the added accumulation of the most abundant sugars (glucose, fructose and sucrose) was measured in the same samples described above. Figure 1B shows that the daily accumulation of sugars in *Arabidopsis* follows a different pattern than starch accumulation. In LD sugar levels were lower previous (Figure 7B above, close circles), than after flowering (open circles), which showed a distinct peak in the middle of the daytime, at ZT8. Interestingly, this pattern was inverse to that observed for starch, which showed a decrease after the floral transition. This effect could also be observed in L*er* (Supplemental Figure 1) and different *Arabidopsis* accessions (Ruiz et al., manuscript in preparation). Thus, it is consistently observed that lower amounts of starch AF are concomitant with higher levels of sugar AF and *vice versa*, higher starch accumulation BF is associated with lower sugar levels BF. When the leaf accumulation of the three major soluble sugars were plotted separately (Figure 1C), glucose was responsible for 60% of the free sugars measured, with lower percentages contributed by fructose (20%) and sucrose (20%).

Sugar levels also varied in SD due to the floral transition, although in this case a continuous high accumulation AF was observed (Figure 1B, lower panel). The increased sugar accumulation in LD and SD AF indicated that the flowering process promoted a mobilization of free sugars in the cell. All these observations suggested that a photoperiod-dependent mechanism modifying plant sugar contents and strongly influenced by the flowering process is present in *Arabidopsis*.

It was also analysed if day length and the floral transition had an influence on transitory starch glycan composition in *Arabidopsis*. Figure 1D shows the amylopectin to amylose ratio measured in mature plant leaves by molecular gel filtration (see methods) in LD (left) or in SD (right) previous (continuous line) and after the floral transition (discontinuous line). The 75% amylopectin / 25% amylose ratio described for *Arabidopsis* starch (Denyer et al., 2001) was only observed in plants grown in LD AF and changed according to the growing condition and reproductive stage of the plant. This way, in LD BF, starch glycan composition ratio was inversed, with amylose fraction reaching 60%, while amylopectin percentage was only 40% of all starch polymers. In SD, the differences were lower and closer to the 70% amylopectin / 30% amylose ratio described for storage starch (Figure 1D right). If anything, amylose proportion in SD BF was slightly reduced compared to SD AF. Therefore, the glycan composition of the granule is also a dynamic characteristic of transitory starch that is not observed for storage starch and reflects the effect of the photoperiod and the reproductive stage of the plant.

Mutant and co-expression analysis correlate *GBSS* expression with carbon mobilization and flowering time

A survey of *Arabidopsis* gene expression microarray experiments in the literature (Ravenscroft, 2005) and databases (Parkinson et al., 2011) identified several genes associated with carbon metabolism that displayed altered expression levels during the floral transition. Among these, the gene *GBSS* (At1g32900) that codes for the *Arabidopsis* putative granule-bound starch synthase, was significantly altered in arrays both overexpressing *CO* and presenting *co* mutations (Romero-Campero et al., 2013). *GBSS* expression is altered by photoperiod in algae and its activity in source tissues is determinant to regulate transitory starch in plants.

The precedent functional, temporal and spatial link between the photoperiod response and *GBSS* expression encouraged a deeper study of this association. For this

reason, *Arabidopsis GBSS* mutant lines from the GABI-Kat (Kleinboelting et al., 2012) and Salk (Alonso et al., 2003) collections were isolated. These lines contain a T-DNA insertion in the predicted *GBSS* transcript from the TAIR genome database (Figure 2A) and were selfed to homozygosis. Employing PCR primers designed to amplify partial *GBSS* transcript fragments from both insertions by RT-PCR, *GBSS* transcript levels indicated that the GABI-Kat line produced 10-20 fold less transcript amount than Col-0 (Figure 2B) and this line was referred thereon as *gbs-1* mutant. The line from the Salk collection showed a smaller decrease in *GBSS* expression (Figure 2B) and was named *gbs-2*. Next, antibodies were raised in rabbit against the recombinant GBSS protein expressed and purified from *E. coli* as described in Methods section (Supplemental Figure 2A). These antibodies were used to perform immunoblots analysis on protein fractions from purified starch granules collected at ZT16 LD, from wild type, *gbs-1* and *gbs-2* mutant plants (Figure 2C). While Col-0 presented a band corresponding to GBSS protein (58 kDa), this band was absent in the *gbs-1* mutant and severely reduced in *gbs-2* mutant.

As GBSS is the only starch synthase able to synthetize amylose in the starch granule, the amylopectin to amylose ratio was measured in mature plants by molecular gel filtration. In Figure 2D, gel filtration column elution profiles of amylopectin and amylose fractions of starch extracted from Col-0 and *gbs* mutants grown in LD BF, are shown. As expected, *gbs-1* and *gbs-2* mutants presented lower amount of amylose than Col-0. Next, the capacity to accumulate starch in the *gbs* mutants compared to Col-0 during 24 h experiments in LD and SD, before and after the floral transition, was measured. Figure 2E left shows that *gbs-1* and *gbs-2* mutants presented a significant reduction in starch accumulation in LD previous to flowering and had lost the difference in starch levels observed in wild type BF and AF (compare with Figure 1A). The overall reduction in starch content in LD in *gbs* mutants was around 20-30% that could account for the loss of the amylose fraction observed in Figure 2D. Starch levels of *gbs* mutants in SD (Figure E right) were very low, and similar to Col-0.

Soluble sugars accumulation in the *gbs* mutant plants was also altered (Figure 2F). This was particularly evident for LD AF because the diurnal peak of sugars at ZT8 observed in Col-0 was absent in the *gbs* mutants (Figure 2F, left). In SD, on the contrary, sugar levels did not show significant differences compared to wild type, remaining constantly low (BF) or constantly high (AF) (Figure 2F, right). As a complementation test, recombinant plants expressing *GBSS* ORF from a 35S promoter (*GBSS*ox) in *gbs-1* and *gbs-2* mutant backgrounds were generated. The *GBSS*ox plants recovered starch content

and the amylopectin / amylose ratio (Supplemental Figure 3). Immunoblots of *GBSS*ox plants revealed a distinct GBSS protein band, although protein levels and activity remained lower than those of wild type plants (Figure 2C and Supplemental Figure 2B-C).

To deepen into the association between starch metabolism and photoperiod observed in *gbs* mutants, we analysed starch and sugars levels in starch excess and starch free *Arabidopsis* mutants. *sex1*, which is defective in starch degradation, accumulates large amounts of starch, (Yu et al., 2001). On the contrary, in the *aps1* mutant plant, which lacks the small catalytic subunit of ADP-glucose pyrophosphorylase, the capacity to produce starch is severely reduced (Ventriglia et al., 2008). In contrast to WT or *gbs* mutants, *sex1* does not show a diurnal pattern of starch accumulation, presenting constant high levels of starch both BF and AF (supplemental Figure 4A). Nevertheless, starch levels were reduced by almost 50% in LD AF indicating that it still retained the capacity to alter starch levels during the floral transition. However, this was not accompanied by a difference in amylopectin / amylose ratio as *sex1* maintained after the floral transition high amounts of amylose compared to amylopectin, similar to Col-0 BF (supplemental Figure 4B). Accordingly to what was observed in other accessions, continuous high levels of starch in the *sex1* mutant were associated to constant low levels of free sugars (supplemental Figure 4C, left) and no difference BF and AF was observed. On the other hand, *aps1* mutant presented negligible amounts of starch in any condition and therefore, continuous high levels of sugars (supplemental Figure 4A and 4C right) independent of the reproductive stage. In *aps1* mutant we were unable to measure the poly-glycan ratio due to the low amounts of starch accumulated. In fact, in a less severe point mutation in the same gene in potato, Lloyd et al. could not identify an amylose fraction in starch from the mutant plant (Lloyd et al., 1999).

The correlation between the floral transition and the mobilization of carbon compounds was confirmed in microarray analysis of several mutants and overexpressing plants grown under different physiological conditions employing the GeneChip *Arabidopsis* ATH1 Genome Array (Affymetrix, Inc). Transcriptional analysis of plants overexpressing *CO* (Simon et al., 1996); *co-10* mutant (Laubinger et al., 2006); *gbs-1* mutant (this work) and *aps1* mutant (Ventriglia et al., 2008), were compared to gene expression profiles from Col-0 plants grown in the absence and presence of 3% (w/v) sucrose. All experimental material was collected at ZT4 (LD condition) from two-week old plants grown in solid agar media. Genes showing a 2-fold difference expression level (down- or up-regulated) were chosen and a correlation analysis between them was

performed. As a result, a gene co-expression network consisting of 3768 genes and 609328 interactions was constructed (Supplemental Figure 5) and graphically represented employing the "organic" layout implemented in the "Cytoscape" software package (Shannon et al., 2003).

The correlation between the expression profile of each gene and physiological data of starch accumulation, soluble sugars and flowering time for the same plants and condition of the microarrays, was integrated into the gene co-expression network. This Genome Wide Associative Study (GWAS) determined two broad different regions within the network: a central domain, which showed a high correlation with sugar and starch accumulation and a peripheral domain, which showed a high correlation with flowering time. This way, the closer the genes are found to the co-expression network centre, the higher the correlation with sugar and starch accumulation while the correlation with flowering time decreases, and *vice versa* (Supplemental Figure 5A, webpage: *http://ackermann.cs.us.es/web_network.html*).

Several algorithms were employed to identify distinct modules within the network integrating the co-expression analysis and the physiological data in the GWAS. Clustering analysis employing the "Partitioning Around Medoids" (PAM) algorithm (Kaufman and Rousseeuw, 1987), identified four optimized distinct groups of functionally related genes including a central cluster corresponding to genes involved in carbon metabolism (Table I and supplemental Figure 5A, blue module). This group comprised genes coding for enzymes involved in starch (*APS1*), trehalose (*TPS8*, *TPS11*) or glycerol (*SRG3*, *GPD*) metabolism, among other metabolic processes. Module 2 (Table I, yellow module) included genes related to photoperiod signalling (*CO*, *FT*, *AGL24*) while module 3 (Table I, red module) included genes involved in response to stimuli such as hormones (*GBF3*, *ARF2*), circadian clock (*LHY/CCA1*, *GI*, *FKF*), light (*CIP1*, *CDF1*, *PHYC*) or cold (*EDF4*, *COR15*). Interestingly, module 4 (Table I, green module) was enriched in genes involved in organic molecules transport, including integral membrane ATPases (*PDR7*, *PGP21*), sugar (*ERD6*, *UTR2*), nucleotides (*DIC1*), nitrogenous substances (*ATTIP2; 3*), sulphate (*SEL1*, *SULTR3; 4*) or potassium (*KAT1*) transporters, among others. The latter included the *GBSS* gene, which suggests that, rather than belonging to a pure carbon metabolic cluster (blue group), *GBSS* is associated with a cluster of genes that connect flowering time with carbon uptake and mobilization (Table I, Supplemental Figure 5B, Supplemental Table II).

Mutations in the *GBSS* gene delays flowering in LD but not in SD

The association between photoperiodic flowering time and *GBSS* expression was further studied analysing its circadian and developmental regulation. First, *GBSS* 24 h expression levels were analysed by Q-PCR and, as reported before in RT-PCR experiments (Tenorio et al., 2003), a circadian expression in LD with a peak level of mRNA accumulation at ZT4 was found (Figure 3A). This peak of expression was moved to the end of the night phase, at ZT0, in plants grown in SD (Figure 3B). The expression of the *GBSS* gene was also lower in LD compared to SD. Both the displacement of the peak of expression from ZT4 in LD to ZT0 in SD and the difference in the mRNA levels, demonstrated a day length influence in *GBBS* expression. In contrast, *gbs-1* and *gbs-2* mutant plants presented minimal *GBSS* mRNA levels in both photoperiods (Figure 3A-B).

We also followed *GBBS* expression during a three-day circadian experiment, the first day in LD and two days in continuous light. *GBSS* mRNA accumulation pattern in Col-0 plants showed a clear circadian influence with maximal expression at ZT4 and minimal expression during the putative dark periods (Figure 3C). A circadian regulation for *GBSS* transcript through the direct binding of CCA1 to its promoter has been reported (Mérida et al., 1999; Tenorio et al., 2003) and this must account for this morning expression peak. However, when levels of *GBSS* mRNA were monitored in LD followed by two consecutive days in continuous dark, the circadian expression of *GBSS* was drastically reduced. This reduction was observed during the consecutive dark days in which *GBSS* morning peak completely disappeared (Figure 3D). These experiments showed that even under a strong circadian control, *GBSS* expression is strongly influenced by light, so that its circadian oscillation is severely altered without light input.

In order to confirm the data obtained in mRNA expression analysis, the presence and activity of GBSS protein were monitored in 24 h course experiments in Col-0. Soluble protein fractions from crude plant extracts, showed no GBSS activity or immunoblot signal (Supplemental Figure 6B). However, when starch granules were isolated and tested for GBSS presence, clear activity and immunoblot signals were detected (Figure 3E-F; Supplemental Figure 6A). This confirmed previous data, based on activity measurements that established the exclusive presence of GBSS inside starch granules and not in soluble, starch-free fractions, as other SSS (Tatge et al., 1999; Zeeman et al., 2002). Starch was then extracted from Col-0 plants grown in LD and SD every 4 h for 24 h to quantify GBSS protein presence. Figure 3E shows GBSS activity and protein accumulation in LD before

and after the floral transition. Protein quantity and activity profiles in LD showed a broad distribution with time: maximum levels at the end of the light period and minimum levels at the end of the night phase. Therefore, GBSS 24 h presence in LD coincides with starch accumulation profile shown in Figure 1A but contrasts with the narrow peak of *GBSS* mRNA at ZT4. This raises an interesting question. If, as suggested (Ral et al., 2006), GBSS protein is progressively incorporated into starch as the granule is synthesized, the narrow peak of mRNA at ZT4 should be sufficient to keep the continuous incorporation of the protein to the starch granule to synthetize the amylose fraction. As amylose is degraded during the night to release sugars for growth and diverse metabolic reactions GBSS protein and activity must slowly decrease. In this scenario, small deviations in *GBSS* expression could have a great influence in starch glycan composition.

In contrast, in SD, the maximum amount of GBSS activity and protein accumulation showed a much narrower time frame, restricted to the daytime and early dark period (Figure 3F). Thus, in SD GBSS is absent during most of the night and would contribute little to starch amylose synthesis. Even more, the increase in GBSS activity and presence observed in LD after the floral transition (Figure 3E, dashed lines) is absent in SD (Figure 3F, dashed lines), strengthening the differences observed between both photoperiods. These differences must be important in the poly-glycan composition of starch and in the capacity to accumulate fixed carbon during the light phase (which will be reflected in sugar release) between both photoperiods. Granules isolated from *gbs* mutant plants showed no GBSS activity or protein presence in 24 h experiments (Supplemental Figure 6C).

The presence of GBSS protein *in vivo* was also followed by monitoring the fluorescence in the confocal microscopy of GBSS:GFP fusions driven by 1kb of the *GBSS* promoter (Figure 3G, Supplemental Figure 7). P*GBSS*:*GBSS*:*GFP* plants complemented the *gbs* mutation restoring GBSS protein presence and activity (Supplemental Figure 2B-C). GBSS:GFP signal was identified inside chloroplasts in starch granules of photosynthetic tissues where it formed organized structures (Supplemental Figure 7A), although it was less organized in a transversal section of the main stem resembling the distribution of phloem tissue (Supplemental Figure 7B). GBSS presence was also detected in tissues with other type of plastids such as the amyloplasts from the layer of columella cells in the main root apical meristem (Supplemental Figure 7B) or in the aleurone layer (Bethke et al., 2007) of developing seeds (Supplemental Figure 7C). GFP signal was detected from very early developmental stages within the siliques until late stages, when

most of the carbohydrate is already converted into oil reserves, expanding the timeframe described for starch presence (Baud et al., 2002; Andriotis et al., 2010). The aleurone layer has been suggested to release sugars to the developing embryo (Penfield et al., 2004). As expected, no GFP presence could be detected in apical meristem tissues of tissues without fully developed chloroplasts (Supplemental Figure 7D). Therefore, the P*GBSS:GBSS:GFP* tissue distribution resembled that described in the literature.

P*GBSS:GBSS:GFP* plants were grown in LD or SD (Figure 3G) and monitored for GFP fluorescence every 4 h. The 24 h accumulation pattern of GBSS:GFP in LD and SD confirmed the data obtained from activity and immunoblot experiments. In LD, GBSS was detected at ZT4 in organized starch granules that increased in density during the daytime and reached their maximum size at the end of the light period (Figure 3G, LD: ZT4-ZT16). During the night the signal gradually disappeared into a low disorganized signal, which was structured again into granules in the following light period (Figure 3G, LD: ZT20-ZT24). In SD this pattern was similar during the light and early dark periods, but was considerably different at the end of the night (Figure 3G SD). Coinciding with the activity and immunoblots in SD, GBSS:GFP signal could not be detected from ZT12 on (Figure 3G SD: ZT12-ZT20) while at this time, a significant amount of GFP presence could still be detected in LD. All together these experiments confirmed the strict association of GBSS to starch granules, its photoperiod dependence and a severely reduced capacity to synthetize amylose in the *gbs* mutants that no other SS could balance.

To test if the differences observed in starch turnover were important for the floral transition, flowering time was scored in wild type and *gbs* mutants in LD and SD (Figure 4 and Supplemental Table I). As observed in Figure 4A-B, *gbs* mutants presented a small but consistent delay in flowering time in LD, repeatedly flowering with 1-2 leaves more (18.6 ± 0.6 for *gbs-1*; 18.9 ± 0.7 for *gbs-2*) than Col-0 (17.4 ± 0.7). Nevertheless, in SD *gbs* mutants and Col-0 plants flowered at the same time (Figure 4C-D). *gbs* mutants in LD displayed more robust rosette leaves and a difference in growth compared to Col-0. In fact, *gbs-1* mutant plants weighed 0.65 ± 0.13 g and *gbs-2* plants 0.60 ± 0.13 g just before flowering while Col-0 plants weighed 0.34 ± 0.07 g. The arrested development could be better appreciated when Col-0 leaves were displayed beside those of *gbs-1* and *gbs-2* mutants (Figure 4E). In turn, in SD, *gbs-1* and *gbs-2* plants were consistently bigger than Col-0 and were slightly retarded early in development, but later flowered at the same time, with no statistically different number of rosette and cauline leaves (Figure 4C-D and Supplemental Table I). *gbs* mutant plants transformed with P*GBSS*:*GBSS*:*GFP* and

35S:*GBSS* constructs complemented the delay in flowering time, reverting to the Col-0 phenotype and even flowering with a consistent, but slightly lower number of leaves, in the case of the GBSSox plants (Supplemental Table I).

Differences in size and flowering time have been described for starch mutants before such as *gigantea* (*gi*), which is known to affect starch and sugar accumulation through a clock-dependent signal (Fowler et al., 1999; Park et al., 1999; Dalchau et al., 2011). As an example, *sex*-1 and *aps1* mutant plants, which displayed a more severe starch metabolic phenotype than *gbs* mutants, showed also a greater delay in floral time in LD (Supplemental Table I, Supplemental Figure 4). Thus, the incapacity to generate a burst of sugars during the floral transition, whether due to lack of starch (*aps1*), or to the impossibility to mobilise sugars from starch (*sex1*), causes an important delay in flowering time. The small delay in flowering time in amylose-less *gbs* mutants is therefore in concordance with the flowering behaviour of other starch metabolic mutants.

Modification of *CONSTANS* expression alters *GBSS* transcript levels

Previous results suggested that the floral transition modified starch accumulation and glycan composition and this could be mediated by GBSS, hence the effect of altering the photoperiod pathway on *GBSS* expression was investigated. *CONSTANS* plays a central role in photoperiodic flowering (Valverde et al, 2004; Jang et al, 2008), thus to verify the effect of *CO* on *GBSS* expression, 35S:*CO*, *co-10* and wild type plants were cultivated in LD or SD and its expression followed during 24 h (Figure 5). In *co-10*, a 20-40% decrease in *GBSS* mRNA levels could be observed at ZT4, the moment of maximum expression in Col-0 in LD (Figure 5A); however, during the rest of the day, the profile remained unmodified. In SD there was no difference in *GBSS* expression, *co-10* showing the same 24 h profile as Col-0 (Figure 5B). So, the decrease in *GBSS* transcript levels in *co* mutant depended on a specific LD signal. This is similar to what has been observed for other *CO* targets such as *FT* (Suárez-López et al., 2001), *SOC1* (Samach et al., 2000) or *TWIN SISTER OF FT* (*TSF*) (Yamaguchi et al., 2005). On the other hand, in 35S:*CO* plants, expression of *GBSS* was drastically altered, showing two-fold higher mRNA amount at ZT4 than wild type plants (Figure 5A). Moreover, at ZT16, when Col-0 shows basal *GBSS* expression levels in LD (Tenorio et al., 2003, Figure 3A) a clear expression peak could be observed in 35S:*CO* plants. This must be attributed to the maximum activity of CO protein that coincides with the end of LD (Suárez-López et al., 2001; Valverde et al., 2004).

Furthermore, ectopic expression of *CO* under the 35S promoter modified the *GBSS* peak of expression at ZT0 in SD (Figure 5B), bringing it to ZT4 as in LD, strongly indicating a direct effect of *CO* on *GBSS* expression. Because CO must be activated by light to promote the expression of its targets, the CO-dependent expression of *GBSS* at ZT16 in SD could not be observed (Figure 5B).

When *GBSS* expression was followed in 35S:*CO* plants for 72 h experiments in LL, *GBSS* mRNA levels gradually increased but still showed a circadian influence (Figure 5C, compare with Figure 3C). Nevertheless, when plants were incubated for two consecutive 24 h dark periods, the signal disappeared the second day (Figure 5D). This indicates that even in the continuous presence of CO, *GBSS* expression needs a strong light input to continue its circadian fluctuation. Known targets of *CO* used as controls such as *FT* gene behaved similarly in these conditions (Supplemental Figure 8A-B).

To confirm the effect of CO on *GBSS* expression and protein presence, 35S:*CO* plants were crossed with the P*GBSS*:*GBSS*:*GFP* construct and grown in LD an SD (Figure 5E). GFP fluorescence was followed on the confocal microscope over a 24 h course. Both LD and SD samples showed an increase in fluorescence compared with Col-0 in all time points except at the beginning of the day (Figure 5E, ZT0 and ZT24). Hence, GBSS abundance in the starch granule was higher during the whole photoperiod both in LD and in SD in the presence of a constitutively expressed CO protein. GFP fluorescence was particularly high in SD if we compare with Figure 3G, when no GFP signal could be detected during the night phase.

The altered presence of GBSS in the granule could change starch amylose composition. Therefore, *co-10* and 35S:*CO* plants were cultivated in LD and SD, samples taken BF and AF and amylopectin / amylose fractions analysed chromatographically. Although *co-10* mutant showed similar starch levels than Col-0 (Supplemental Figure 8E) no change in amylose composition BF and AF was observed (Figure 6A). This strongly supports that, the change in starch amylose composition observed previously during the floral transition in LD (Figure 1D, left), must be due to CO activity. Similarly, there was no difference in amylose fraction in SD, BF and AF (Figure 6B) when CO is not active in SD (Suárez-López et al., 2001). Thus, the similarity in Col-0 and *co-10* amylopectin / amylose ratio in SD (Figure 1D, right) should be attributed to CO inactivity. On the contrary, in 35S:*CO* plants in LD, where we had observed an induction of *GBSS* mRNA presence and GBSS stability, starch amount was reduced 1/10 compared to Col-0 (Supplemental Figure 8E) but in this residual amount, the amylose fraction reached very

high levels. This difference was enhanced after the floral transition, presenting the highest amylose fraction observed in any plant or condition studied (Figure 6C). Amylose levels were also high in 35S:*CO* plants in SD, but no significant difference could be observed BF and AF (Figure 6D).

To test if *GBSS* mRNA levels were naturally altered by CO due to the flowering process, Col-0, *co-10* and *gbs-1* plants were grown on soil in SD for 5 weeks and then transferred to LD. This change in photoperiod promotes a strong an immediate flowering signal in Col-0. RNA samples were collected daily for four days at ZT4 and ZT16, and the levels of *GBSS* were measured by Q-PCR (Figure 6E). In Col-0 plants, the day previous to LD exposure, *GBSS* showed the regular pattern of expression of a plant growing in SD (Figure 3B): low expression at ZT4 and no expression at ZT16 (Figure 6E, dark grey columns). During the first day in LD, although the ZT4 peak did not increase, a new *GBSS* mRNA peak at ZT16 appeared. However, in the second day exposed to LD the *GBSS* morning peak drastically increased and the evening peak still remained high. In the third day, the peak of expression at ZT4 was now predominant, while the ZT16 peak almost disappeared, having the plant adapted to the new LD condition (compare with Figure 3A). This pattern was drastically altered in the *co-10* mutant, which showed a very small increase in *GBSS* expression at ZT16 in all days and who responded very poorly to the SD to LD transition (Figure 6E, light grey columns). *gbs-1* mutant was used as a negative control and did not show any *GBSS* expression in any phase of the experiment (Figure 6E, white columns). Analysis of *FT* expression in wild type as a control in the same experiment (Figure 6F) demonstrated that the activation of *GBSS* in the SD to LD transition was occurring in a similar pattern as the *FT* expression described in the literature (Fornara et al., 2010). Thus, as in the case of *FT* expression, that is activated by *CO* only in the presence of light (Suárez-López et al., 2001), the peak of *GBSS* mRNA dependent on CO activity at ZT16 was absent in SD, but could be observed at a maximum of ZT16 in LD. When *FT* expression was measured in *co-10* mutant plants, no increase in mRNA levels was observed, as previously described (Corbesier et al., 2007). The *gbs-1* line (light grey) behaved as a wild type plant for *FT* expression. Consequently, the increase in the morning peak of *GBSS* expression and the presence of the ZT16 peak observed in the transition from SD to LD are transitory effects that are not present in the *co* mutant, confirming a natural *CO*-dependent influence on *GBSS* expression in the floral transition.

To provide further evidence of *GBSS* activation by *CO*, inducible 35S:*CO-GR* plants (Simon et al., 1996) that promote CO nuclear import and activity upon

dexamethasone (DEX) addition, were employed. Plants were grown in agar plates in LD, DEX was added at ZT0 and *GBSS* mRNA followed by Q-PCR every 2 h for 20 h after the drug treatment (Figure 7A, left). *FT* expression was used as a positive control of the experiment (Figure 7A, right). An increase in *GBSS* expression (+ DEX) was detected 2 h after treatment, kept at maximum expression after 8 h and showed a second small expression peak at 16 h, quickly decreasing during the dark period. *FT* expression was also increased by the DEX treatment, although it reached maximum levels at the end of the light period and also quickly decreased after dark. When cycloheximide (CHX), a potent inhibitor of protein synthesis, was added at ZT0 (+ DEX + CHX), *GBSS* expression remained at levels similar to + DEX, indicating that expression of *GBSS* by *CO* does not need intermediate factors.

These experiments suggested that CO could be inducing *GBSS* expression during the morning and the evening, in a different way than *FT*, which shows a maximum of expression at the end of the day. *GBSS* promoter, considering 2 kb upstream the predicted ATG codon (Supplemental Figure 9A), presents several putative CO-complex binding sites (Wenkel et al., 2006; Tiwari et al., 2010) that could mediate the direct binding of CO protein to *GBSS* promoter. To test this hypothesis, chromatin immunoprecipitation (ChIP) experiments on *GBBS* promoter were performed. We employed ChIP grade commercially available antibodies (Sigma) in nuclear extracts from 35S:*CO*:TAP-TAG plants (see methods) grown in LD and collected during the early morning (ZT1) and late evening (ZT16) during the floral transition. Indeed, enrichment on two different fragments of the *GBSS* promoter could be observed (Figure 9B-C) that confirmed the direct induction of *GBSS* expression by CO. The *GBSS* promoter site 1 enriched in the ChIP experiments at ZT16 (Figure 7C) included a putative target for the HEME ACTIVATOR COMPLEX (HAP) binding site (supplemental Figure 9A), where complex CO-HAP has been also shown to bind on the *FT* promoter (Wenkel et al., 2006). In fact, a control experiment on *FT* promoter employing the same 35S:*CO*:TAP-TAG nuclear extracts at ZT16 confirmed a ChIP enrichment on site 4, which contains a HAP conserved sequence (Supplemental Figure 9B). On the other hand, an enrichment on ZT1 sample (Figure 7B) was found in site 3 of *GBSS* promoter corresponding to a CORE (CO Responsive Element) sequence that has also been reported as a direct binding site for CO through its CCT domain (Tiwari et al., 2010) but no enrichment was observed for the binding site found at ZT16. We also confirmed binding of CO to a CORE site in *FT* promoter at ZT16 (target 5, Supplemental Figure 9B). In samples at ZT1 we did not detect any significant binding of CO-TAP-TAG

protein to the *FT* promoter. Thus, binding of CO to *GBSS* promoter takes place at different sites in the morning and in the evening, while on the *FT* promoter the binding sites were detected only in the evening samples.

Finally, the association between *CO* and *GBSS* was confirmed employing a genetic approach. *gbs* mutants were crossed to plants overexpressing *CO* under the ubiquitous 35S promoter and the phloem-specific SUC2 promoter (Imlau et al., 1999) with the aim of finding an epistatic effect on flowering time. 35S:*CO* plants flowered with 6.1 ± 0.7 leaves in LD while plants crossed to either *gbs-1* or *gbs-2* mutant alleles delayed flowering to 9.1 ± 0.8 and 9.5 ± 1.2 leaves, respectively (Table II). Furthermore, SUC2:*CO* plants flowered with 6.3 ± 0.8 leaves in LD, while *gbs* mutations in the background delayed flowering of SUC2:*CO* plants to 8.1 ± 0.9 leaves for *gbs-1* and 8.0 ± 0.8 leaves for *gbs-2* (Table II). Therefore, *GBSS* mutations had a much stronger effect on plants overexpressing *CO* than on the wild type (Figure 4A, Table II), supporting a strong epistatic effect on its flowering phenotype. The delayed flowering on SUC2:*CO* plants that specifically express CO in the phloem was further evidence that the epistasis of *GBSS* on *CO* was taking place in the specific tissue were CO has been proposed to induce its florigenic activity (An et al., 2004).

DISCUSSION

Transitory starch accumulation depends on day length and is influenced by GBSS activity

Part of the success of plants confronting changing environmental conditions they cannot evade is to present a narrow association between developmental and metabolic processes through their life cycle (Eveland and Jackson, 2011). The metabolic response of a plant to unexpected changes in the environment is extremely fast and is fine-tuned to the needs of the plant at every stage of its developmental program (Nicotra et al., 2010; Pyl et al., 2012). This confers plants a highly plastic adaptation to the environment (Casal et al., 2004). Reproductive behaviour is impinged by this response and many metabolic signals activate or inhibit the floral transition (Amasino, 2010, Eveland and Jackson 2011, Wahl et al, 2013). On the other hand, during developmental transitions, plants must adapt their metabolism and metabolite transport to respond to the energetic and structural needs of the new tissues and structures created. In the floral transition the effect of different sources of carbon, nitrogen, phosphorous or sulphur intake are well documented (Bernier et al., 1993; Schulze et al., 1994). Nevertheless, the regulatory mechanisms associated to these changes remain obscure to date.

The floral transition can be considered a repressed state that under the correct stimuli is triggered to induce the reproductive stage (Boss et al., 2004). One of the most important stimuli, due to its wide inter-specific distribution and evolutionary conservation (Valverde et al., 2011), is the photoperiod signalling pathway. A photoperiod-dependent mechanism in *Arabidopsis* triggers the production of the florigenic signal in source tissues (leaves) that is transported through the phloem to the sink tissues (meristems) inducing the reproductive stage (Fornara et al., 2010). In this transition, the effect of carbohydrates such as sugars has been previously reported (Corbesier et al., 1998; King et al., 2008). Here, we provide sound evidences that support that carbon partitioning in *Arabidopsis* leaves changes during the transition to flowering and that this is accompanied by drastic changes in the amylose composition of starch granules (Figure 1 and Supplemental Figure 1). Besides, we describe that the same photoperiod signal that activates *FT* expression through the central regulator CO is simultaneously responsible for the mobilization of sugars employing a similar mechanism. We further propose that this action is exerted through a starch synthase which is a unique and most peculiar among SS. GBSS is inside the starch

granule, is the only responsible for amylose synthesis and has a direct influence on starch glycan composition and, therefore, on the capacity to accumulate and mobilize sugars from it (Streb and Zeeman, 2012). Although a great number of regulatory genes affecting the floral transition have already been described, the effect of a structural gene in the flowering process and the mechanism by which a final metabolic effect is achieved, have rarely been described before.

GBBS has been recently connected in a co-expression network with a cluster of genes including two *CO-like* genes (Ingkasuwan et al., 2012, Romero-Campero et al., 2013). The mutation of these genes had a clear effect on *GBSS* expression. When we tested the effect of these mutations and of some other *COL* genes on *GBSS* expression, the normal circadian expression of *GBSS* was altered (data not shown) suggesting that a photoperiodic signal is normally controlling *GBSS* mRNA levels. Control of starch synthetizing enzymes by genes related to light or the circadian clock has been described before (Mérida et al., 1999; Streb and Zeeman 2012). Actually, expression of *GBSS* in *Antirrhinum* and *Arabidopsis* is controlled directly by the association of CCA1 (a central clock transcription factor) to *GBSS* promoter, thus explaining its morning peak of expression at ZT4 (Tenorio et al., 2003). The clock is altered by photoperiodic signals so it is clear that such peak of expression of *GBSS* differs from LD (ZT4) to SD (ZT0) (see Figure 3). Indeed, the effect of different photoperiod dependent COLs may account for this peak of expression, suggesting an interesting link between clock core genes such as *CCA1*/*LHY* and *COLs* in the control of morning genes.

The ratios of amylopectin to amylose composition in starch described here depend on photoperiod and developmental signals and are consistent with the observations of the changes in GBSS protein levels shown in Figure 3 and supplemental Figure 6. Our cytological studies further suggest that there is a crucial time, early in the day, for *GBSS* mRNA transcription and incorporation of the protein inside the new-formed granules. This equilibrium is displaced to the light phase (ZT4) in LD compared to the peak in the night (ZT0) for SD, probably reflecting a different developmental role for both photoperiod-modified starches. We have also shown that small variations in the quantity of *GBSS* transcript or in its circadian pattern of expression (as the ZT16 peak mediated by CO in the floral transition), have a strong influence on starch composition. On the other hand, there seems to be a direct link between the capacity to liberate sugars from starch and the activity associated to GBSS, probably due to both disequilibrium in the starch composition and increased facility to liberate sugars from it. Therefore, high amylose starch generated

by GBSS induction could be an optimal source of sugars to fuel the energetic demands associated to the floral transition. It has also been suggested that an increase in the source to sink sugar flow through the phloem may help transport the florigenic substances, such as FT, that trigger the floral response in the meristem (Corbesier et al., 1998). Moreover, the activation of phloem- and meristem-specific sugar transports associated with the flowering process (Matsoukas et al., 2012) strongly suggests this effect. This way, we propose a mechanism by which *CO* alteration of *GBSS* expression may help to liberate large amount of sugars during the floral transition (Figure 8).

Photoperiod signals control development and carbon metabolism in plants

It was recently described that starch accumulation and *GBSS* expression are under circadian (Mérida et al., 1999; Ral et al., 2006) and photoperiod control (Serrano et al., 2009). This suggests that photosynthetic eukaryotes have developed a narrow control of starch accumulation depending on light signals (Gibon et al., 2009; Streb and Zeeman, 2012). In general terms, light input is never altered in a more drastic way than by day length in temperate climates. The CO homolog of *Chlamydomonas* alters starch accumulation (Serrano et al., 2009) and in this paper we show that in *Arabidopsis* such alteration can be performed by the GBSS-dependent modification of starch glycan composition. During the activation of the floral transition in *Arabidopsis*, which can be mimicked by transferring SD-grown plants to LD (Corbesier et al., 2007), *FT* expression is induced by CO activity. Our results show that a natural mobilization of sugars, concomitant with a change of transitory starch accumulation and glycan composition, as well as a shift in the *GBSS* pattern of expression, also takes place (Figure 1 and Figure 3). We have further demonstrated that this activation is greatly impaired in a *co* mutant background, identifying *CONSTANS* as a necessary agent for *GBSS* induction and, thus, for the modification of transitory starch composition during the floral transition (Figure 5). Indeed, an extra peak of *GBSS* expression at ZT16 could be observed during the floral transition that quickly disappeared after three days in LD, when the peak of *GBBS* expression reverted to the single clock-dependent peak at ZT4 (see Figure 6). As this effect cannot be observed in the *co-10* mutant, neither can it be reproduced in SD in 35S:*CO*, when CO protein is inactive, our observations point out to a specific CO-dependent process. On the other hand, modification of *CO* mRNA levels has a clear effect on starch

accumulation, amylopectin / amylose composition and sugar content (Figure 6 and supplemental Figure 8D-F), which is most notable during the floral transition, suggesting a general photoperiod control of carbon mobilization during the flowering process.

Because of the observed activation of *GBSS* expression in different *CO* overexpression lines (35S:*CO*, 35S:*CO*:*GR* and 35S*:CO:TAP-TAG)* and because we have detected by ChIP experiments that CO protein presence in *GBSS* promoter is enriched in flowering conditions, we think that activation of *GBSS* expression by CO is direct (Figure 7). Therefore, we propose that during the floral transition a peak of *CO* expression and activity is directly involved in *GBSS* expression and this is associated to drastic changes in starch composition and sugar release (Figure 8). Amounts of sugar accumulated through GBSS-induced modification of starch granules can then be channelled through the phloem and accompany FT to enhance its florigenic function. It has also been suggested that a burst of sucrose induces the production of trehalose-6-phosphate, and this could induce flowering (Sulpice et al., 2013; Wahl et al., 2013). In our co-expression analysis genes involved in the transport of organic substances correlated with the flowering process. Nevertheless, whether this effect is direct, through the activation of other target genes, or by simply enhancing the phloematic movement of substances from source to sink tissues, cannot be inferred by our experiments. What this work strongly supports is that sugar mobilization does occur during the floral transition (Bernier et al., 1993) and it has a photoperiodic component (Matsoukas et al., 2012). What is more, CO is directly involved both in the progression of the florigen and in sugar mobilization through the altered expression of *FT* (An et al., 2004) and *GBSS* genes (this work), respectively. The effect of *CO* on *GBSS* may be one of the mechanisms implicated in the coordination of photoperiod induction of flowering and carbon mobilization. Modification of the photoperiod perception by the plant may then be employed to modify starch glycan composition and mobilization, promoting sugar release with diverse biotechnological applications.

Finally, as this photoperiod regulation of GBSS activity can also be found in *Chlamydomonas*, this finding strongly suggest that an ancient photoperiod regulatory module controlling sugar mobilization existed in unicellular green algae. This module expanded and diversified to alter and interconnect other physiological processes through different COLs (Romero-Campero et al., 2013), such as the reproductive transition due to the effect of CO.

Supplemental Data

The following materials are available in the online version of this article:

- Supplemental Experimental Procedures

- References to supplemental data.

- Supplemental Tables:

Supplemental table I. Flowering time in LD and SD of wild type and recombinant plants.

Supplemental table II. GO categories significantly enriched in the different modules identified in the gene co-expression network.

Supplemental table III. List of primers employed in this work.

- Supplemental Figures:

Supplemental Figure 1. Changes of starch and soluble sugars in Col-0 and L*er* ecotypes during the floral transition.

Supplemental Figure 2. Complementation analysis of *gbs-1* mutant.

Supplemental Figure 3. Purification of recombinant GBSS. Activity and protein levels in different recombinant plant lines.

Supplemental Figure 4. Starch composition and sugar content in starch less and starch excess mutants.

Supplemental Figure 5. Gene co-expression analysis.

Supplemental Figure 6. Presence of GBSS protein in different conditions and plant lines.

Supplemental Figure 7. Distribution of GBSS:GFP fusion protein in P*GBSS*:*GBSS*:*GFP* (*gbs-1*) plants resembles wild type GBSS distribution.

Supplemental Figure 8. Changes in *CO* expression alter *FT* mRNA levels, starch and sugars accumulation.

Supplemental Figure 9. *GBSS* promoter and Binding of CO to *FT* promoter.

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AUTHOR CONTRIBUTIONS

M. I. O-M., T. A. co-designed and performed the experimental work, E. L.-R., F. E. S., F. J. R.-C., B. C., and M. T. R. performed cellular and bioinformatics experiments. J. M. R. and F.V. coordinated, designed the experimental work and wrote the paper.

FIGURE LEGENDS

- Figure 1. Starch and sugar contents in *Arabidopsis* are modified by the photoperiod and developmental stage. A. Starch content in Col-0 during 24 h either in LD (black) or SD (grey) conditions before (BF, continuous lines) and after the floral transition (AF, dashed lines). Leaves samples were taken every 4 h, considering Zeitgeber time 0 (ZT0) when lights are switched on. **B**. Whole content of major sugars (glucose, fructose and sucrose) in Figure 1A samples in LD (upper panel) and in SD (lower panel). **C**. Glucose (top), fructose (middle) and sucrose (bottom) concentration in the same samples as A in LD. **D.** Gel filtration elution profile of semi-hydrolysed starch granules from wild type Col-0 in different photoperiods (LD at ZT16, left; SD at ZT8, right) and developmental stages (BF and AF) showing the amylopectin and amylose fractions. In all experiments data from three biological replica ± s.e.m. are shown. Significant differences (t-student test) between Col-0 BF and Col-0 AF, is indicated by asterisks: *P<0.05, **P<0.01 and ***P<0.001.

- Figure 2. Characterization of *gbs* mutants. A. Structure of *Arabidopsis GBSS* gene. Exons are indicated by boxes (ORF white; 5', 3' UTR grey), introns by lines. T-DNA insertion sites in the GABI (*gbs-1*) and SALK (*gbs-2*) lines are marked by inverted triangles. **B.** PCR amplification of *GBSS* cDNA fragments employing primers shown in A. Amplification of *UBQ10* cDNA was used as a control. **C.** Detection of GBSS protein with specific antibodies in protein extracts from Col-0, *gbs-1*, *gbs-2* and *GBSS* over-expressor (in *gbs-1* background) plants. Quantification of the chemiluminescence signal of each band relative to *gbs-1* is shown above. The apparent Molecular Mass is shown on the left. **D.** Amylopectin-amylose composition of starch granules from Col-0 (black), *gbs-1* (dark grey) and *gbs-2* (light grey) plants grown in LD, harvested at ZT16 BF. Notice the absence of amylose fraction in both mutant lines. **E.** Leaf starch content in Col-0 (black), *gbs-1* (dark grey) and *gbs-2* (light grey) mutants before flowering (BF, solid lines) and after flowering (AF, dashed lines) in plants grown in LD (left) and SD (right). **F.** Sugar content in Col-0 (black), *gbs-1* (dark grey) and *gbs-2* (light grey) before flowering (BF, solid lines) and after flowering (AF, dashed lines) in plants grown in LD (left) and SD (right). Data represent three biological replica ± s.e.m. Significant differences (t-student test) between Col-0 and *gbs* lines, is indicated by asterisks: *P<0.05 and **P<0.01.

- Figure 3. Photoperiodic expression of *GBSS* mRNA and protein. A. 24 h expression profile of *GBSS* gene in wild type Col-0, *gbs-1* and *gbs-2* mutants in LD BF. **B.** As in A but in SD. **C.** *GBSS* mRNA levels in Col-0 24 h LD BF followed by 48 h in continuous light (LL). **D.** *GBSS* mRNA levels in Col-0 24 h LD BF followed by 48 h in continuous dark (DD). RNA samples were taken from the same equivalent leaves and data are the replica of at least three biological samples ± s.e.m. cDNA was amplified by Q-PCR. **E.** Protein presence by immunoblot quantification (black) and activity (grey) levels in a 24 h circadian experiment in LD BF (solid lines) and AF (dashed lines). **F.** As in E but in SD. G. P*GBSS*:*GBSS*:*GFP* (*gbs-1)* plants grown in LD (upper panel) or SD (lower panel) monitored during 24 h BF under the confocal microscope at 4 h intervals. Images show chloroplasts of leaf parenchyma cells with GFP fluorescence in green and chlorophyll fluorescence in red.

- Figure 4. Flowering phenotype of *gbs* mutants. A. Number of leaves (rosette black, cauline, grey) at the moment of flowering of wild type, *gbs-1* and *gbs-2* mutants in LD. **B.** Col-0, *gbs-1* (middle) and *gbs-2* (right) plants grown in LD. **C.** Number of leaves (rosette dark grey, cauline light grey) at the moment of flowering of wild type, *gbs-1* and *gbs-2* mutants in SD. **D.** Col-0, *gbs-1* (middle) and *gbs-2* (right) plants grown in SD. **E.** Phenology of wild type and *gbs* mutants in LD. Data are the mean of scoring at least ten plants including s.e.m.

- Figure 4. Effect of CO on *GBSS* expression and presence and stability of GBSS protein in 35S:*CO in vivo*. A. *GBSS* expression levels by Q-PCR during 24 h in Col-0 (dark continuous line, circles), *co-10* (grey dotted line, triangles) and 35S:*CO* (dark dashed line, squares) plants grown in LD BF. **B.** As in A with plants grown in SD BF. **C.** *GBSS* mRNA levels in 35S:*CO* plants in 24 h LD BF followed by 48 h in continuous light (LL). **D.** *GBSS* mRNA in 35S:*CO* plants in 24 h LD BF followed by 48 h in continuous dark (DD). RNA samples were taken from the same equivalent leaves and data are the replica of at least three biological samples ± s.e.m. cDNA was amplified by Q-PCR. **E.** 35S:*CO* P*GBSS*:*GBSS*:*GFP* plants grown in LD (upper panel) or SD (lower panel) monitored during 24 h under the confocal microscope at 4 h intervals. Images show chloroplasts of leaf parenchyma cells with GFP fluorescence in green and chlorophyll fluorescence in red.

- **Figure 5. Altering CO expression affects starch glycan composition and *GBSS* expression during the floral transition. A.** Amylopectin-amylose fractions in *co-10* plants BF (solid line) and AF (dashed line) in LD. **B.** As in A, in SD. **C.** Amylopectin-amylose fractions in 35S:*CO* plants BF (solid line) and AF (dashed line) in LD. **D.** As in C, in SD. **E.** *GBSS* expression levels at ZT4 and ZT16 measured by Q-PCR in Col-0 (dark grey columns), *co-10* (light grey columns) and *gbs-1* (white columns) plants grown for five weeks in SD and transferred to LD for three days. **F.** Control expression of *FT* in the experiment in E. Data are the media of at least three biological replica including s.e.m.

- **Figure 6. Direct induction of *GBSS* expression by CO. A**. *GBBS* (left) and *FT* (right) expression levels measured by Q-PCR in 35S:*CO*:*GR* (*co-2 tt4*) plants after the addition of dexamethasone (+ DEX: continuous line, full circles); without DEX addition (- DEX: dotted line, full diamonds) and with DEX plus cycloheximide (+ DEX + CHX: dark dashed line, empty circles). DEX was added at time 0 h (ZT0, LD) and samples were taken thereon every 2 h for 20 h. In all cases data are the media of three biological replica ± s.e.m. expressed in relative units compared to *UBQ10* levels. **B.** Chromatin immunoprecipitation (ChIP) analysis of 35S:*CO*:TAP-TAG and control plants collected at ZT1 at the moment of the floral transition. **C**. Chromatin immunoprecipitation (ChIP) analysis of 35S:*CO*:TAP-TAG and control plants collected at ZT16 at the moment of the floral transition. The black line represents 1922 bp of *GBSS* promoter 5' of the predicted starting ATG. Putative target sites and ORF negative control site (#7) employed for PCR amplification are identified in the table and marked by numbers (see Supplemental Table III and Supplemental figure 9). Above each number, columns (grey for Col-0, black for 35S:*CO*:TAP-TAG plants) representing the fold amplification enrichment (Y axis scale) compared to the control by Q-PCR for each site are shown.

- **Figure 7. Model for the photoperiodic regulation of *GBSS* expression**. The cartoons represent *GBSS* mRNA (blue) and protein (red) levels in LD (above) and SD (below) during a 24 h course. Both before flowering (BF, left) and after flowering (AF, right) scenarios are depicted. The arrows indicate the diverse *GBSS* expression peaks observed in different developmental stages: At ZT4 due to the influence of the photoperiod and circadian clock in LD; at ZT0 due to the circadian clock in SD and at ZT16 in LD during

the floral transition due to the effect of CO. The green flux diagram in LD AF represents the proposed burst of sugars generated due to the starch modification caused by CO-GBSS action during the floral transition.

TABLES

Table I

Module	Number of genes	Functional Annotation	GO Term	P-value	Genes	TAIR ID
Blue	1824	metabolic process	GO:0008152	1.88×10^{-6}	*APS1*	At5g48300
		biosynthetic process	GO:0009058	3.97×10^{-6}	*TPS8*	At1g70290
		primary metabolic process	GO:0044238	1.27×10^{-5}	*GPD*	At5g40610
					SRG3	At3g02040
Green	733	oxidation-reduction process	GO:0055114	2.28×10^{-5}	*ERD6*	At1g08930
		lipid transport	GO:0006869	4.73×10^{-4}	*UTR2*	At4g23010
		organic substance transport	GO:0071702	9.30×10^{-4}	*PDR7*	At1g15210
					GBSS	At1g32900
Yellow	302	regulation of metabolic process	GO:0019222	4.03×10^{-4}	*CO*	At5g15840
					FT	At1g65480
		regulation of gene expression	GO:0010468	9.90×10^{-4}	*AGL24*	At4g24540
					SEU	At1g43850
Red	949	response to stimulus	GO:0050896	6.81×10^{-4}	*GBF3*	At2g46270
		response to UV	GO:0010224	6.81×10^{-4}	*GI*	At1g22770
					CIP1	At5g41790
					EDF4	At1g13260

Table I. GO terms significantly enriched in the modules of the gene co-expression network. Each module is associated with a colour code according to the gene co-expression network (Supplemental Figure 5) and the number of genes in each module is presented. Functional annotation and GO terms were inferred using Bioconductor and the GOrilla software (Supplemental data). Statistically sound P-values are given for each GO term significantly present in each module (Supplemental Table II). Some representative genes for each module are listed providing their three-letter identifiers and TAIR codes.

Table II

Plant	Days to flower	Leaves number
Col-0	21.0 ± 1.5	17.4 ± 0.7
gbs-1	21.0 ± 1.0	18.6 ± 0.6 *
gbs-2	21.0 ± 0.8	18.9 ± 0.7 *
35S:*CO*	11.0 ± 1.2	6.1 ± 0.7
35S:*CO gbs-1*$^+$	12.1 ± 1.5	9.1 ± 0.8 **
35S:*CO gbs-2*$^+$	12.3 ± 1.8	9.5 ± 1.2 **
SUC2:*CO*	10.5 ± 0.7	6.3 ± 0.9
SUC2:*CO gbs-1*$^+$	14.3 ± 1.0	8.1 ± 1.2 *
SUC2:*CO gbs-2*$^+$	14.00 ± 0.9	8.0 ± 1.3 *

Table II. **Effect of *gbs* mutation in plants overexpressing *CO* in LD.** The name of single or double recombinant plants is given on the left column. Flowering time was scored as days to flower (middle column) and by total number of leaves at the moment of appearance of the flower bud (right column). For single recombinant and wild type plants, data are the media of scoring at least 10 plants ± s.e.m. **+** For double recombinant plants, three different lines were chosen, at least ten plants were scored for each line and the media was calculated for all plants including s.e.m. Significant differences (t-student test) between Col-0 and *gbs* lines, 35S:*CO* and 35S:*CO gbs* lines and SUC2:*CO* and SUC2:*CO gbs lines* is indicated by asterisks: *P<0.05 and **P<0.01.

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