Photoperiodic Control of Carbon Distribution during the Floral Transition in Arabidopsis

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Flowering is a crucial process that demands substantial resources. Carbon metabolism must be coordinated with development through a control mechanism that optimizes 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 (Cr-CO) in the control of the photoperiod response in the green alga Chlamydomonas reinhardtii and its influence on starch metabolism was demonstrated. In this work, we show that transitory starch accumulation and glycan composition during the floral transition in Arabidopsis thaliana are regulated by photoperiod. Employing a multidisciplinary approach, we demonstrate a role for CO in regulating the level and timing of expression of the GRANULE BOUND STARCH SYNTHASE (GBSS) gene. Furthermore, we provide a detailed characterization of a GBSS mutant involved in transitory starch synthesis and analyze its flowering time phenotype in relation to its altered capacity to synthesize amylose and to modify the plant free sugar content. Photoperiod modification of starch homeostasis by CO may be crucial for increasing the sugar mobilization demanded by the floral transition. This finding contributes to our understanding of the flowering process.

INTRODUCTION

The plant life cycle is strongly influenced by environmental conditions, which affect the plant’s capacity to obtain energy for growth and development (Nicotra et al., 2010). The floral transition is a crucial developmental decision for a plant because falling to produce a reproductive signal at the correct time of the year seriously limits its capacity to yield descendants, and, for this reason, this process is strictly regulated (Casal et al., 2004). Based on an 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 and generate 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 pathway, and light, through the CONSTANS (CO)-FLOWERING LOCUS T (FT) module, are key external conditions that influence the floral 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 (Ohoto et al., 2001). Recently, roles for Suc-dependent kinases (Baena-González et al., 2007) and trehalose-6-phosphate (Gómez et al., 2010; Wahl et al., 2013) in vegetative growth and flowering have been suggested, while a role for INDETERMINATE DOMAIN8 in the activation of Suc synthase and its involvement in photoperiodic flowering has also been described (Seo et al., 2011). It is surprising then that, given that sugars have such an important role in flowering time, there is still no indication of 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 the amount of

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storage starch is much more stable. The starch biosynthetic pathway has been extensively studied in diverse species, and considerable progress has been made toward 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 catalyzed by ADP-Glc pyrophosphorylase, starch synthase, starch branching enzyme, and starch debranching enzyme (Ball and Morell, 2003; Zeeman et al., 2010). Starch synthases (SSs) transfer the glucosyl group of ADP-Glc, the product of ADP-Glc pyrophosphorylase, to the nonreducing 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 and granule-bound starch synthases (GBSSs).

GBSS is exclusively located in starch granules (Sivak et al., 1993). It was originally identified in maize (Zea mays) 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 glycogen (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 (Solanum tuberosum) (Dry et al., 1992; van der Steege et al., 1992), pea (Pisum sativum) (Dry et al., 1992), barley (Hordeum vulgare) (Rohde et al., 1988), wheat (Triticum aestivum) (Clark et al., 1991), snapdragon (Antirrhinum majus; 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 performed 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 Suc provides one of the early signals for the induction of flowering (Bernier et al., 1993).

Analysis of Arabidopsis starch-less mutants has shown that starch synthesis 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; Rai et al., 2006). GBSS expression in Chlamydomonas reinhardtii is also under photoperiodic control, so that Cr-CO, an ortholog of Arabidopsis CO, directly influences Cr-GBSS 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 C. reinhardtii (Romero and Valverde, 2009; 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 article, we show that amylose constitutes a dynamic polymer within transitory starch whose turnover depends on the photoperiodic regulation of GBSS. In fact, gbs mutant display a small but consistent delay in flowering time exclusively in LD and coexpression 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 daylength is regulated by CO through the modification of GBSS expression during the floral transition. Hence, by altering GBSS expression, the photoperiod pathway coordinates the florigenic signal through FT with the burst of sugars needed to drive the flowering process. This mechanism may reflect an evolutionarily conserved photoperiodic signaling 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

To assess the effect of daylength on the production of transitory starch, we measured starch accumulation in the leaves of Arabidopsis Columbia-0 (Col-0) plants grown in 16 h light and 8 h darkness (long day [LD]) and 8 h light and 16 h darkness (short day [SD]) over 24-h courses. As we were interested in establishing if the starch accumulation pattern (Lu et al., 2005; Gibon et al., 2009) was influenced by the floral stage of the plant, we measured the amount of plant starch in Arabidopsis leaves every 4 h for 24 h in LD and SD, 2 d before and 2 d 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; closed circles) starch accumulation reached higher levels (up to one-third more at ZT16, significant according to a Student’s t test; P < 0.001) than after flowering (AF; open circles). This effect could be observed in both Col-0 and Landsberg erecta (Supplemental Figure 1) as well as in a number of other Arabidopsis accessions (M.T. Ruiz, M.I. Ortiz-Marchena, F.J. Romero-Campero, F.X. Picó, F. Valverde, and J.M. Romero, unpublished data). 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 (Glc, Fru, and Suc) 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 conditions, sugar levels were significantly (P < 0.05) lower BF (Figure
than AF (open circles), the latter showing a distinct peak in the middle of the daytime, at ZT8, that was absent BF. Interestingly, this pattern was inverse to that observed for starch, which showed a decrease after the flowering transition. This effect could also be observed in Landsberg erecta (Supplemental Figure 1; P < 0.001) and different Arabidopsis accessions (M.T. Ruiz, M.I. Ortiz-Marchena, F.J. Romero-Campero, F.X. Picó, F. Valverde, and J.M. Romero, unpublished data). Thus, it is consistently observed that lower amounts of starch AF are concomitant with higher levels of sugar AF and vice versa (i.e., that higher starch accumulation BF is associated with lower sugar levels BF). When the leaf accumulation of the three major soluble sugars was plotted separately (Figure 1C), Glc was responsible for 60% of the free sugars measured, with lower percentages contributed by Fru (20%) and Suc (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, bottom panel). The increased sugar accumulation in LD and SD AF indicated that the flowering process promoted 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.

We also examined if daylength 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) conditions before (continuous line) and after (discontinuous line) the floral transition. The 75% amylopectin/25% amylose ratio described for Arabidopsis

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 (gray) conditions BF (continuous lines) and AF (dashed lines). Leaf samples were taken every 4 h, considering Zeitgeber time 0 (ZT0) when lights are switched on. fw, fresh weight.

(B) Whole content of major sugars (Glc, Fru, and Suc) in samples from (A) in LD (top panel) and in SD (bottom panel).

(C) Glc (top), Fru (middle), and Suc (bottom) concentration in the same samples as (A) in LD (scale of the y axis is reduced to visualize the differences).

(D) Gel filtration elution profile of semihydrolyzed 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 replicates ± se are shown. Significant differences (Student’s t test) between Col-0 BF and Col-0 AF are marked by asterisks: *P < 0.05, **P < 0.01, and ***P < 0.001.
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 the 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, the amylose proportion in SD BF was slightly reduced compared with SD AF. Therefore, the glycan composition of the granule is also a dynamic characteristic of transitory starch that reflects the effect of the photoperiod and the reproductive stage of the plant.

**Mutant and Coexpression Analysis Correlate GBSS Expression with Carbon Mobilization and Flowering Time**

A survey of Arabidopsis gene expression microarray experiments in the literature (Ravenstクロ, 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, GBSS 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 influences transitory starch synthesis in plants.

The functional, temporal, and spatial link between the photoperiod response and GBSS expression encouraged a deeper study of this association. For this reason, we isolated Arabidopsis GBSS mutant lines from the GABI-Kat (Kleinboelting et al., 2012) and Salk (Alonso et al., 2003) collections. These lines contain a T-DNA insertion in the predicted GBSS transcript from The Arabidopsis Information Resource (TAIR) genome database (Figure 2A) and were selfed to homozygous state. Using PCR primers designed to amplify partial GBSS transcript fragments from both insertions by RT-PCR, we found that the GABI-Kat line produced 10- to 20-fold less transcript than did Col-0 (Figure 2B), and this mutant line is hereafter referred to as gbs-1. 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 recombinant GBSS expressed and purified from Escherichia coli as described in Methods (Supplemental Figure 2A). These antibodies were used to perform immunoblot 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 (58 kD), this band was absent in gbs-1 mutant and severely reduced in gbs-2.

As GBSS is the only starch synthase able to synthesize 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 amounts of amylose than Col-0. Next, the capacity to accumulate starch in the gbs mutants compared with 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 BF (P < 0.01) and had lost the difference in starch levels observed in the wild type BF and AF. The overall reduction in starch content in LD in gbs mutants was around 20 to 30%, which could account for the loss of the amylose fraction observed in Figure 2D. Starch levels of gbs mutants in SD (Figure 2E, right) were very low and similar to Col-0.

Soluble sugar accumulation in gbs 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 with the wild type, remaining constantly low (BF) or constantly high (AF) (Figure 2F, right). As a complementation test, recombinant plants expressing GBSS open reading frame (ORF) from a 35S promoter (P35S: GBSS) in gbs-1 and gbs-2 mutant backgrounds were generated. The P35S:GBSS plants recovered starch content and the amylose fraction (Supplemental Figures 2B and 2C). Immunoblots of P35S:GBSS plants revealed a distinct GBSS protein band, although protein levels and activity remained lower than those of wild-type plants (Figure 2C; Supplemental Figures 2D and 2E).

To confirm the association between starch metabolism and photoperiod observed in gbs mutants, we analyzed 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-Glc pyrophosphorylase (APS1), the capacity to produce starch is severely reduced (Ventriglia et al., 2008). In contrast with the wild type 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 3A). 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 with amylopectin, similar to Col-0 BF (Supplemental Figure 3B). Accordingly to what was observed in other accessions, continuous high levels of starch in the sex1 mutant were associated with constant low levels of free sugars (Supplemental Figures 3A, left, and 3C) and no difference BF and AF was observed. On the other hand, the aps1 mutant presented negligible amounts of starch in any condition and, therefore, continuous high levels of sugars (Supplemental Figures 3A and 3C, right) independent of the reproductive stage. In the aps1 mutant, we were unable to measure the polyglycan ratio due to the low amounts of starch accumulated. In fact, in transgenic antisense plants of a homologous gene in potato, Lloyd et al. (1999) detected a reduction in the amylose fraction in starch from the recombinant plants.

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). 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 with gene expression profiles from Col-0 plants grown in the absence and presence of
3% (w/v) Suc. All experimental material was collected at ZT4 (LD conditions) from 2-week-old plants grown in agar media. Genes showing a 2-fold difference in expression level (down- or upregulated) were chosen and a correlation analysis between them was performed. As a result, a gene coexpression network consisting of 3768 genes and 609,328 interactions was constructed (Supplemental Figure 4) 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 coexpression network. This Genome Wide Associative Study 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
coexpression network center, the higher the correlation with sugar and starch accumulation, while the correlation with flowering time decreases, and vice versa (Supplemental Figure 4A). This observation is also presented in the network deposited on the website: https://viridiplantae.ibvf.csic.es/web_network/web_network.html.

Several algorithms were employed to identify distinct modules within the network integrating the coexpression analysis and the physiological data in the Genome Wide Associative Study. 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 1, blue module; Supplemental Figure 4A). This group comprised genes encoding enzymes involved in starch (AP51), trehalose (TREHALOSE-PHOSPHATE SYNTHASE8 [TPS8] and TPS11) or glucuronol (SARENCE-RELATED GENE3 [SRG3], GLYCEROL-3-PHOSPHATE DEHYDROGENASE [GPD]) metabolism, among other metabolic processes. Module 2 (Table 1, yellow module) included genes related to photoperiod signaling (CO, FT, and AGAMOUS-LIKE24 [AGL24]), while module 3 (Table 1, red module) included genes involved in responses to stimuli such as hormones [BOX FACTOR3 [GBF3] and AUXIN RESPONSE FACTOR2 [ARF2]], the circadian clock [LATE ELONGATED HYPOCOTYL [HY] CIRCADIAN CLOCK ASSOCIATED1 [CCA1], GIANT ANTEA [GI], and FLAVIN BINDING, KELCH REPEAT, F BOX1], light [COP1 INTERACTING PROTEIN1 [CIP1], CYCLING DOF FACTOR1 [CDN1], and PHYTOCHROME C], or cold [ETHYLENE RESPONSE DNA BINDING FACTOR4 [EDF4] and COLD-REGULATED GENE15]. Interestingly, module 4 (Table 1, green module) was enriched in genes involved in organic molecule transport, including integral membrane ATPase [PSEUDOTROPIC DRUG RESISTANCE7 [PDR7], P-GLYCOPROTEIN21], sugar [EARLY-RESPONSE TO DEHYDRATION PROTEIN6 [ERD6] and UDP-GALACOSATE TRANSPORTER2 [UTR2]], acid [DICARBOXYLATE CARRIER], nitrogenuous substance [TOMOLAST-PROTEIN2 [ATTIP2] and ATTIP3]), sulfate [SLENATE-RESISTANCE1, SULFATE TRANSPORTER3 [SULT3], and SULT4], or potassium [POTASSIUM CHANNEL IN ARABIDOPSIS THALIANA1] transporters, among others. The latter included GBSS, 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 1; Supplemental Figure 4B and Supplemental Table 1).

**Mutations in GBSS Delay Flowering in LD but Not in SD**

The association between photoperiodic flowering time and GBSS expression was further studied to analyze its circadian and developmental regulation. First, GBSS 24-h expression levels were analyzed by quantitative PCR (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). GBSS expression was also lower in LD compared with 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 daylength influence in GBSS expression. By contrast, gbs-1 and gbs-2 mutant plants presented minimal GBSS mRNA levels in both photoperiods (Figures 3A and 3B).

We also followed GBSS expression during a 3-d circadian experiment, the first day in LD and 2 d in continuous light (LL). The GBSS mRNA accumulation pattern in Col-0 plants showed a clear circadian influence with maximal expression at ZT4 and minimal expression during the dark period (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 darkness (DD), the circadian expression of GBSS was drastically reduced. This reduction was observed during the consecutive dark days in which the GBSS morning peak completely disappeared (Figure 3D). These experiments showed that even under strong circadian regulation, GBSS expression is strongly influenced by light, so that its circadian oscillation is severely altered in the absence of light input.

To confirm the data obtained in the mRNA expression analysis, the presence and activity of GBSS 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 5B). However, when starch granules were isolated and tested for the presence of GBSS, clear activity and immunoblot signals were detected (Figures 3E and 3F; Supplemental Figure 5A). 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 SSSs (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 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, the 24-h presence of GBSS in LD coincides with the 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 (Ralley et al., 2006), GBSS 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 synthesize 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.

By 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. Furthermore, 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 the two photoperiods. These differences must be important in the
polyglycan 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 5C).

The presence of GBSS in vivo was also followed by monitoring the fluorescence in the confocal microscopy of GBSS:GFP (green fluorescent protein) fusions driven by 1 kb of the GBSS promoter (Figure 3G; Supplemental Figure 6). PGBSS:GBSS:GFP plants complemented the gbs mutation restoring GBSS protein presence and activity (Supplemental Figures 2D and 2E). GBSS:GFP signal was identified inside chloroplasts in starch granules of photosynthetic tissues where it formed organized structures (Supplemental Figure 6A), although it was less organized in a transversal section of the main stem resembling the distribution of phloem tissue (Supplemental Figure 6B). GBSS was also detected in tissues with other types of plastids, such as the amyloplasts from the layer of columella cells in the main root apical meristem (Supplemental Figure 6C). Therefore, the PGBSS:GBSS:GFP tissue distribution resembled that described in the literature (Streb and Zeeman, 2012).

PGBSS: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 to 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 to 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, the GBSS:GFP signal could not be detected from ZT12 onwards (Figure 3G; SD: ZT12 to ZT20), while at this time interval, a significant amount of GFP could still be detected in LD. Together, these experiments confirmed the strict association between GBSS and starch granules, its photoperiod dependence, and a severely reduced capacity to synthesize amyllose 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 the wild type and gbs mutants in LD and SD (Supplemental Figure 7A and Supplemental Table 2). As observed in Supplemental Figures 7A and 7B, gbs mutants presented a small but significant (P < 0.05) and consistent delay in flowering time in LD, repeatedly flowering with one to two 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 (Supplemental Figures 7C and 7D). gbs mutants in LD displayed more robust rosette leaves and a difference in growth compared with 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 (Supplemental Figure 7E). 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 (Supplemental Figures 7C and 7D and Supplemental Table 2). gbs mutant plants transformed with PGBSS:GBSS:GFP and P35S: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 P35S:GBSS plants (Supplemental Table 2).

Differences in size and flowering time have been described for starch mutants before, such as gi, which is known to affect

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### Table 1. GO Terms Significantly Enriched in the Modules of the Gene Coexpression Network

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<td>1.27 × 10⁻⁵</td>
<td>GDP</td>
<td>At5g40610</td>
</tr>
<tr>
<td>Green</td>
<td>733</td>
<td>Oxidation-reduction process</td>
<td>GO:0055114</td>
<td>2.28 × 10⁻⁵</td>
<td>SRG3</td>
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<td></td>
<td></td>
<td>Lipid transport</td>
<td>GO:0006869</td>
<td>4.73 × 10⁻⁴</td>
<td>ERD6</td>
<td>At1g88930</td>
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<tr>
<td></td>
<td></td>
<td>Organic substance transport</td>
<td>GO:0071702</td>
<td>9.30 × 10⁻⁴</td>
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<td>At4g23010</td>
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<tr>
<td>Yellow</td>
<td>302</td>
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<td>GO:0019222</td>
<td>4.03 × 10⁻⁴</td>
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<td></td>
<td></td>
<td>Regulation of gene expression</td>
<td>GO:0010468</td>
<td>9.90 × 10⁻⁴</td>
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<td>At1g32990</td>
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<tr>
<td>Red</td>
<td>949</td>
<td>Response to stimulus</td>
<td>GO:0050896</td>
<td>6.81 × 10⁻⁴</td>
<td>CO</td>
<td>At5g15840</td>
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<td></td>
<td></td>
<td>Response to UV</td>
<td>GO:0010224</td>
<td>6.81 × 10⁻⁴</td>
<td>FT</td>
<td>At1g65480</td>
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</tbody>
</table>

Each module is associated with a color code according to the gene coexpression network (Supplemental Figure 4), and the number of genes in each module is presented. Functional annotation and Gene Ontology (GO) terms were inferred using Bioconductor and the GOrilla software (see Methods). Statistically sound P values are given for each Gene Ontology term significantly present in each module (Supplemental Table 1). Some representative genes for each module are listed, along with their three-letter identifiers and TAIR codes.
starch and sugar accumulation through a clock-dependent signal (Fowler et al., 1999; Park et al., 1999; Dalchau et al., 2011). As an example, sex1 and aps1 mutant plants, which displayed a more severe starch metabolic phenotype than gbs mutants, also showed a greater delay in floral time in LD (Supplemental Table 2 and Supplemental Figure 3D). 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 mobilize 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 behavior of other starch metabolic mutants.

Modification of CO 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, we investigated the effect of altering the photoperiod pathway on GBSS expression. Because CO plays a central role in photoperiodic flowering (Valverde et al., 2004; Jang et al., 2008), to verify the effect of CO on GBSS expression, we cultivated 35S:CO, co-10, and wild-type plants in LD or SD and monitored their expression over a 24-h period (Figure 4). In co-10, a 20 to 40% decrease in GBSS mRNA levels could be observed at ZT4, the moment of maximum expression in Col-0 in LD (Figure 4A); however, during the rest of the day, the profile remained unmodified. In SD, there was no difference in GBSS expression, with co-10 showing the same 24-h profile as Col-0 (Figure 4B). So, the decrease in GBSS transcript levels in the 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), SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1 (SOC1) (Samach et al., 2000), or TWIN SISTER OF FT (Yamaguchi et al., 2005). On the other hand, in 35S:CO plants, expression of GBSS was drastically altered, showing a 2-fold higher mRNA amount at ZT4 than wild-type plants (Figure 4A).

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 is attributed to the maximum activity of CO 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 4B), 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 4B).

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 (cf. Figures 4C and 3C). Nevertheless, when plants were incubated for two consecutive 24-h dark periods, the signal disappeared the second day (Figure 4D). 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, behaved similarly in these conditions (Supplemental Figures 8A and 8B). To confirm the effect of CO on GBSS expression and protein presence, 35S:CO plants were crossed with the PGBSS:GBSS:GFP construct and grown in LD and SD (Figure 4E). GFP fluorescence was followed by confocal microscopy 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 4E, 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 were taken BF and AF, and amylopectin/amylose fractions were analyzed chromatographically. Although the co-10 mutant showed similar starch levels than Col-0 (Supplemental Figure 8E), no change in amylose composition BF and AF was observed (Figure 5A). This strongly suggests 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 5B) 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, the amount of starch was reduced compared with Col-0 (Supplemental Figure 8E), but in this small 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 5C). Amylose levels were also high in 35S:CO plants in SD, but no significant difference could be observed BF and AF (Figure 5D).

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 and immediate flowering signal in Col-0. RNA samples were collected daily for 4 d at ZT4 and ZT16, and the levels of GBSS were measured by q-PCR (Figure 5E). 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 5E, dark-gray 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 remained high. In the third day, the peak of expression at ZT4 was now predominant, while the ZT16 peak almost disappeared, as the plant had adapted to the new LD condition (compared 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 which responded poorly to the SD to LD transition (Figure 5E, light-gray columns). The gbs-1 mutant was used as a negative control and did not show any GBSS expression in any phase of the experiment (Figure 5E, white columns). Analysis of FT expression in the wild type as a control in the same experiment (Figure 5F) demonstrated that the activation of GBSS in the SD to LD transition occurred 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 gray) 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 6A, left). FT expression was used as a positive control of the experiment (Figure 6A, right). An increase in GBSS expression (+DEX) was detected 2 h after treatment, reached 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 induce GBSS expression during the morning and the evening, in a different way than FT, which shows maximum expression at the end of the day. The GBSS promoter, encompassed in the 2-kb region upstream of 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 to the GBSS promoter. To test this hypothesis, we performed chromatin immunoprecipitation (ChIP) experiments on the GBBS promoter. We
employed ChIP-grade commercially available antibodies (Sigma-Aldrich) 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 (Figures 6B and 6C) that confirmed the direct induction of GBSS expression by CO. The GBSS promoter site 1 enriched in the ChIP experiments at ZT16 (Figure 6C) included a putative target for the HEME ACTIVATOR COMPLEX (HAP) binding site (Supplemental Figure 9A), where complex CO-HAP has also been shown to induce 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 ChIP enrichment on site 4, which contains a HAP-conserved sequence (Supplemental Figure 9B). On the other hand, an enrichment on the ZT1 sample (Figure 6B) was found in site 3 of the 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 the CORE site in the FT promoter at ZT16 (target 5; Supplemental Figure 9B). In samples at ZT1, we did not detect any significant binding of CO-TAP-TAG to the FT promoter. Thus, binding of CO to the GBSS promoter takes place at different sites in the morning and in the evening, while binding sites on the FT promoter were detected only in the evening samples.

**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), but in SD. (C) Amylopectin-amylose fractions in 3SS:CO plants BF (solid line) and AF (dashed line) in LD. (D) As in (C), but in SD. (E) GBSS expression levels at ZT4 and ZT16 measured by Q-PCR in Col-0 (dark gray columns), co-10 (light gray columns), and gbs-1 (white columns) plants grown for 5 weeks in SD and transferred to LD for 3 d. (F) Control expression of FT in the experiment in (E). Data are the means of at least three biological replicates including SE.
Finally, the association between CO and GBSS was confirmed using 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 2). 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 2). Therefore, GBSS mutations had a much stronger effect on plants overexpressing CO than on the wild type (Supplemental Figure 7A; Table 2), supporting a strong epistatic effect on its flowering phenotype. The delayed flowering of SUC2:CO plants that specifically express CO in the phloem was further evidence that the epistasis of GBSS on CO occurred in the specific tissue where CO has been proposed to induce its florigenic activity (An et al., 2004).

**DISCUSSION**

Transitory Starch Accumulation Depends on Daylength and Is Influenced by GBSS Activity

Due to their sessile lifestyle, plants have evolved a versatile metabolism that adapts to the changing environmental conditions in every developmental stage (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.,...

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**Figure 6. Direct Induction of GBSS Expression by CO.**

**(A)** GBSS (left) and FT (right) expression levels measured by Q-PCR in 35S:CO:GR (co-2 tt4) plants upon the addition of DEX (+DEX: continuous line, filled circles), without DEX addition (-DEX: dotted line, filled diamonds), and with DEX plus CHX (+DEX + CHX: dark dashed line, empty circles). DEX was added at time 0 h (ZT0, LD), and samples were then taken every 2 h for 20 h. In all cases, data are the means of three biological replicates ± SE expressed in relative units compared with UBQ10 levels.

**(B)** ChiP analysis of 35S:CO:TAP-TAG and control plants collected at ZT1 at the moment of the floral transition.

**(C)** ChiP analysis of 35S:CO:TAP-TAG and control plants collected at ZT16 at the moment of the floral transition. The black line along the x axis represents 1922 bp of the 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 (Supplemental Table 3 and Supplemental Figure 9). Above each number, columns (gray for Col-0 and black for 35S:CO:TAP-TAG plants) representing the fold amplification enrichment (y axis scale) compared with the control by Q-PCR for each site are shown. Data are the means of three biological replicates including SE.
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 many regulatory genes affecting the floral transition have already been described, to our knowledge, the effect of a structural gene on flowering and the mechanism by which a final metabolic effect is achieved have not been described before.

GBSS was recently connected in a coexpression network with a cluster of genes including two CO-like (COL) genes and CCA1 (Ingkasuwan et al., 2012; Romero-Campero et al., 2013). The mutation of these two COL genes had a clear effect on GBSS expression (Ingkasuwan et al., 2012) that we confirmed in our experiments, suggesting that a photoperiodic signal normally regulates GBSS mRNA levels. Control of starch synthesizing enzymes by genes related to light or the circadian clock has been described before (Mérida et al., 1999; Streb and Zeeman, 2012). Actually, the expression of GBSS in snapdragon and Arabidopsis is regulated directly by the association of CCA1 (a central clock transcription factor) with the 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 a peak of expression of GBSS differs from LD (ZT4) to SD (ZT0) (Figure 3). The effect of different photoperiod-dependent COLs may account for this peak in expression, suggesting an interesting link between clock core genes, such as CCA1/LHY and COLs in the regulation of morning genes. Our data suggest that binding of CO to the GBSS promoter differs from binding to the FT promoter in two senses. First, CO probably binds in the morning in a complex with other COL genes at the site seen at ZT1 (CORE element) and this could imply the collaboration of the clock through the CCA1/LHY activity, as the site described for CCA1 binding (Tenorio et al., 2003) is close but does not overlap with that of CO. In fact, CO shows a peak of stability in the morning (Valverde et al., 2004) once the light degradation mediated by COP1 is over and the light degradation of CO dependent on HOS1 and probably PHYB (Lázaro et al., 2012) has not yet taken over the degradation of CO. This binding of CO to the GBSS promoter actually cannot be observed in ChIP experiments with samples collected at ZT6. Second, binding of CO through the CCAAT box binding complex (Wenkel et al., 2006) to the GBSS promoter at the HAP sites occurs only during the evening exclusively during the transition to flowering (except in plants overexpressing CO). Therefore, our data suggest that binding of CO to its targets could be mediated by a HAP complex during the flowering transition and through other COLs and the effect of the clock in the morning (CORE site).

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 5. 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 newly formed granules. This equilibrium is displaced to the light phase (ZT4) in LD compared with the peak in the night (ZT0) in 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

<table>
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<tr>
<th>Plant</th>
<th>Days to Flower</th>
<th>Leaf Number</th>
</tr>
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<tbody>
<tr>
<td>Col-0</td>
<td>21.0 ± 1.5</td>
<td>17.4 ± 0.7</td>
</tr>
<tr>
<td>gbs-1</td>
<td>21.0 ± 1.0</td>
<td>18.6 ± 0.6*</td>
</tr>
<tr>
<td>gbs-2</td>
<td>21.0 ± 0.8</td>
<td>18.9 ± 0.7*</td>
</tr>
<tr>
<td>35S:CO</td>
<td>11.0 ± 1.2</td>
<td>6.1 ± 0.7</td>
</tr>
<tr>
<td>35S:CO gbs-1a</td>
<td>12.1 ± 1.5</td>
<td>9.1 ± 0.6**</td>
</tr>
<tr>
<td>35S:CO gbs-2a</td>
<td>12.3 ± 1.8</td>
<td>9.5 ± 1.2**</td>
</tr>
<tr>
<td>SUC2:CO</td>
<td>10.5 ± 0.7</td>
<td>6.3 ± 0.9</td>
</tr>
<tr>
<td>SUC2:CO gbs-1a</td>
<td>14.3 ± 1.0</td>
<td>8.1 ± 1.2*</td>
</tr>
<tr>
<td>SUC2:CO gbs-2a</td>
<td>14.00 ± 0.9</td>
<td>8.0 ± 1.3*</td>
</tr>
</tbody>
</table>

The name of single or double recombinant plants is given in the left column. Flowering time was scored as days to flower (middle column) and by total number of leaves at the moment of the appearance of the first flower bud (right column). For single recombinant and wild-type plants, data are the means of scoring at least 10 plants ± se. Significant differences (determined using Student’s t test) between Col-0 and gbs lines, 35S:CO and 35S:CO gbs lines, and SUC2:CO and SUC2:CO gbs lines are marked by asterisks: *P < 0.05 and **P < 0.01.

*aFor double recombinant plants, three different lines were chosen, at least 10 plants were scored for each line, and the means was calculated for all plants including se.

2010; Pyl et al., 2012). This allows plants to be highly plastic in their response to the environment (Casal et al., 2004). Reproductive behavior 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 sulfur intake are well documented (Bernier et al., 1993; Schulze et al., 1994). Nevertheless, the regulatory mechanisms associated with these changes remain obscure to date.

The floral transition can be considered as 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 interspecific distribution and evolutionary conservation (Valverde, 2011), is the photoperiod signaling 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 show 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; Supplemental Figures 1 and 2).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 SSs. GBSS is inside the starch granule, is the only SS responsible for amylose synthesis,
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 with GBSS, probably due to both a disequilibrium in the starch composition and an 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 with the floral transition. It has also been suggested that an increase in the source to sink sugar flow through the phloem may help transport 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 transporters associated with the flowering process (Matsoukas et al., 2012) strongly suggests this effect. Based on these findings, we propose a mechanism by which CO alteration of GBSS expression may help to liberate large amounts of sugars during the floral transition (Figure 7).

Photoperiod Signals Regulate 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 tight control of starch accumulation depending on light signals (Gibon et al., 2009; Streb and Zeeman, 2012). Generally, light input is never altered in a more drastic way than by daylength in temperate climates. The CO homolog of C. reinhardtii alters starch accumulation (Serrano et al., 2009), and in this article, 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 (Figures 1 and 3). We have further demonstrated that this activation is greatly impaired in a co mutant background, identifying CO as a necessary agent for GBSS induction and, therefore, for the modification of transitory starch composition during the floral transition (Figure 4; Supplemental Figure 8E). Indeed, an extra peak of GBSS expression at ZT16 could be observed during the floral transition that quickly disappeared after 3 d in LD, when the peak of GBBS expression reverted to the single clock-dependent peak at ZT4 (Figure 5E). As this effect cannot be observed in the co-10 mutant, nor can it be reproduced in SD in 35S:CO, when CO is inactive, our observations suggest the existence of a specific CO-dependent process. On the other hand, modification of CO mRNA levels has a clear effect on starch accumulation, amylpectin/amylose composition, and sugar content (Figure 5; Supplemental Figures 8D to 8F), 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 detected by ChIP experiments that the presence of CO in the GBSS promoter is enriched in flowering conditions, we think that activation of GBSS expression by CO is direct (Figure 6). Therefore, we propose that during the floral transition a peak in CO expression and activity is directly

![Figure 7. Model for the Photoperiodic Regulation of GBSS Expression.](image)

The cartoons represent GBSS mRNA (blue) and protein (red) levels in LD (above) and SD (below) during a 24-h course. Both BF (left) and 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 and 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.
involved in GBSS expression and that this is associated with drastic changes in starch composition and sugar release (Figure 7). The amount of sugar accumulated through GBSS-induced modification of starch granules can then be channeled through the phloem and accompany FT to enhance its florigenic function. It has also been suggested that a burst of Suc induces the production of trehalose-6-phosphate and that this could induce flowering (Wahl et al., 2013; Sulpice et al., 2014). In our coexpression 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 from 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 (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 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 C. reinhardtii, this finding strongly suggests that an ancient photoperiod regulatory module that regulated sugar mobilization existed in unicellular green algae. This module expanded and diversified to alter and interconnect other physiological processes through different COs (Romero-Campero et al., 2013), such as the reproductive transition due to the effect of CO.

**METHODS**

**Plant Material and Growth Conditions**

Plants were grown in controlled cabinets on peat-based compost (for starch, sugar, and amyllose determinations) or in Murashige and Skoog plates (for Q-PCR assays). Seeds were previously incubated for 4 d at 4°C in the dark before sowing under a 16-h-light/8-h-dark cycle (LD) or a 8-h-light/16-h-dark cycle (SD) with temperatures ranging from 22°C (day) to 18°C (night). For experiments involving light quantity changes, LD-entrained plants were moved to separate growth chambers with 24-h-light (LL), 24-h-dark (DD), or other photoperiods. Leaf samples were harvested at the time intervals indicated in each case (i.e., 2 d BF and 2 d AF for starch and sugar analysis). For Q-PCR assays, plant material was harvested 10 d after sowing in MS agar plates.

**Starch Analysis**

Starch granules were extracted by a modification of the method described by Hubert (1983). Samples were washed with hot 80% (v/v) ethanol for 2 h at 80°C and then washed with the same solution at room temperature until the tissue was free of pigments. Particles including starch were pelleted by centrifugation at 15,000 g for 10 min at 4°C, suspended in 1 mL of 0.2 M KOH, and incubated at 100°C for 30 min. After incubation, pH was adjusted to 5.0 with 300 µL of 1 N acetic acid solution. Then, 35 µL of B-amylase (from porcine pancreas, Sigma-Aldrich) solution (7.4 units in 0.1 M Na acetate buffer, pH 4.5) was added to samples, and the samples were incubated at 37°C for 30 min. To each sample, 165 µL of amyloglucosidase (from Aspergillus oryzae; Sigma-Aldrich) solution (5 units in 0.1 M Na acetate buffer, pH 4.5) was added, and the tubes were incubated at 55°C for 2 h. After digestion, the tubes were incubated at 100°C for 2 min and centrifuged, and the Glc in the supernatant was analyzed enzymatically using hexokinase and Glc-6-P dehydrogenase (see below; Jones et al., 1977).

**Amylopectin/Amylose Analysis**

Starch granules were isolated with extraction buffer (50 mM HEPES and 1% [v/v] Triton-X 100, pH 7.6) and filtered; the samples were pelleted by centrifugation and suspended in 2 mL of 50% (v/v) Percoll. After suspension, the samples were centrifuged and the pellet was washed with absolute ethanol. Particles including starch were pelleted by centrifugation as before and then suspended in 1 mL of 1 M NaOH; samples were incubated at 100°C for 1 h. After boiling, tubes were centrifuged and the supernatant recovered. Routine separation of amyllopectin and amylose was performed as described by Zeeman et al. (2002) using a Sepharose CL2B column (40-cm length, 1-cm cross-sectional area, and 30-ml volume), and 0.5-ml fractions were collected at a rate of one fraction per 1 min. Starch (2 mg) was dissolved in 500 µL of 0.5 M NaOH, applied to the column, and eluted with 10 mM NaOH. Fractions were adjusted to pH 5.0 by the addition of a small volume of acetic acid 0.1 M pH 4.5, and the glyc an content was measured as described above.

**Determination of Soluble Sugars**

Sugars were quantified by two methods. In the first method, modified from Sonnewald et al. (1991), leaf samples were taken at the indicated time points and extracted with 80% (v/v) ethanol in 10 mM HEPES-KOH, pH 7.7, at 80°C for 2 h. The supernatant was used to determine Glc, Fru, and Suc content (Stitt et al., 1989) by the sequential addition of 5 units of Glc-6-P dehydrogenase, 5 units of hexokinase and Glc-6-P isomerase, and 20 units of invertase and measuring 340-nm absorbance (reduction of NAD+) at different intervals.

In a second method, sugars were identified and quantified employing a high-performance anion-exchange chromatography protocol. In short, soluble plant extracts as above were purified using nylon filters (GE Healthcare) diluted in running buffer and samples run through a CarboPac PA-10 4 × 250-mm column (ThermoFisher) with pulsed amperometric detection (Dionex). Final peaks were quantified using Chromelon software (v. 7.0; ThermoFisher) and sugar standards of known concentrations.

**DNA, RNA, and Bioinformatics Analysis**

**RNA Isolation**

RNA was isolated from Arabidopsis thaliana seedlings employing the Trizol (Invitrogen) protocol as described by the manufacturer. Leaf samples (0.1 g) were ground in liquid nitrogen and the powder mixed with 1 mL of Trizol and 0.2 mL of chloroform; the mixture was then centrifuged at 16,000 g for 15 min at 4°C. The supernatant was treated with 1 volume of 2-propanol, incubated for 15 min at room temperature, and centrifuged at 16,000 g for 10 min at 4°C. The pellet was resuspended in 1 mL of 3 M LiCl and incubated for t>10 min at room temperature and centrifuged at 16,000 g for 10 min at 4°C. The pellet was washed with 70% (v/v) ethanol and centrifuged at 16,000 g for 10 min at 4°C. The final RNA sample was suspended in 30 µL of diethylpyrocarbonate-treated water and quantified employing a ND-1000 Spectrophotometer (NanoDrop).

For microarray hybridization, Trizol-extracted RNA from three independent sets of plate-grown seedlings (fully expanded cotyledons) was further purified using an RNeasy Mini Kit (Qiagen) according to the kit
manual. Affymetrix Gene Chip array expression profiling was performed at the Centro Nacional de Biotecnología–Consejo Superior de Investigaciones Científicas service, Madrid, Spain. The raw data were processed using the LIMMA algorithm (Wettenhall and Smyth, 2004). The Classification SuperViewer software (Zhu et al., 2003) was used to ascribe functions to the up- and downregulated genes.

**Real-Time Q-PCR**

One microgram of Trizol-isolated RNA was used to synthesize cDNA employing the Quantitec Reverse Kit (Qiagen) following the instructions recommended by the manufacturer. cDNA was diluted to a final concentration of 10 ng/µL and stored at −20°C until Q-PCR was performed. Primers used to amplify the 3’untranslated region of GBSS, FT, and UBP10 (Supplemental Table 3) as reference genes were designed with the Oligo analyzer program (Integrated DNA Technologies). Q-PCR was performed in an iQTMS multicolor real-time PCR detection system from Bio-Rad in a 10-µL reaction: primer concentration 0.2 µM, 10 ng of cDNA, and 5 µL of SensiFAST TM SYBR and the Fluorescein kit (Bioline). Each sample was measured at least in triplicate. The Q-PCR program consisted of (1) one cycle (95°C, 2 min), (2) 40 cycles (95°C, 5 s; 60°C, 10 s; and 72°C, 6 s), and (3) one cycle (72°C, 6 s). Fluorescence was measured at the end of each extension step, and the melting curve was performed between 55 and 95°C. The initial concentration of candidate and reference genes was calculated by means of LingRegPCR software version 11.0 (Ruijter et al., 2009). Normalized data were calculated by dividing the average of at least three replicates of each sample from the candidate and reference genes.

**Microarrays Analysis and Coexpression Network Generation**

The Affymetrix microarray data were preprocessed using the R package affy (Gautier et al., 2004) for quality control, background correction, normalization, and calculation of the expression levels, employing the RMA algorithm. Differentially expressed genes were selected according to a fold-change criterion (LIMMA R package) using a threshold of 2 when comparing the expression level in a given mutant with that in the Col-0 ecotype. Gene correlations based on the gene expression profiles were calculated using the Pearson correlation coefficient within the WGCNA R package (Zhang and Horvath, 2005). A cutoff threshold of 0.95 was chosen to generate our coexpression network since this produced a good fit to a power-law distribution and scale-free topology. These significant gene interactions were imported into Cytoscape version 2.8.2 and visualized using the Organic layout.

**Clustering and Functional Enrichment**

The correlation between gene profiles was used as a distance to determine different clusters in the coexpression network. Optimal clustering algorithm and number of clusters were determined using a combination of internal, stability, and functional criteria, such as the Dunn index and the average proportion of nonoverlap. The R package clValid (Brock et al., 2008) was used to validate this clustering analysis by comparing different algorithms such as hierarchical clustering, K-means, PAM, self-organizing tree algorithm, and fuzzy analysis together with different numbers of clusters. The three best choices of a clustering algorithm and number of clusters was PAM with two, three, and four modules. In order to decide the optimal number of clusters, physiological data representing the starch content, sugar content, and flowering time of the sampled plants were integrated in our network calculating the correlation between them and the expression profile of the genes in each cluster. This revealed that the combination of the clustering algorithm PAM with four modules produced the most biologically informative clustering. To assign potential biological functions to each module, Gene Ontology term enrichment analysis was performed using the R packages annotate, annaify, and ath1121501.db in Bioconductor (Gentlemen et al., 2004) and the software GOrilla (Eden et al., 2009). The gene coexpression network can be explored using the Web-based tool available in at https://vividplantea.ibiv.fsic.es/web_network/web_network.html.

**Statistical Analysis**

The data presented in the figures are reported as the sample mean ± SD. The statistical significance of the pairwise comparisons between the means of different samples was performed using a Student’s t test assuming normally distributed data sets with equal variances. The null hypothesis that the means are equal was rejected when contrasted against the alternative hypothesis that the mean of one of the data sets is greater than the other one. The difference between the sample means was considered statistically significant with a P value lower than 0.05 (marked with a single asterisk), 0.01 (marked with two asterisks), or 0.001 (marked with three asterisks). This statistical analysis was performed using the R statistical programming language. Specifically, the function t test from the stats R package was used (R Core Team, 2013).

**Protein Techniques**

**Purification of Recombinant Nt-(His)6GBSSI**

A cDNA fragment encoding Arabidopsis GBSS in pBS plasmid was obtained from the Riken Institute. The GBSS cDNA fragment was amplified by PCR using specific primers that excluded the plastid-transit peptide (predicted by the TargetP1.1 program) and including a Met residue, as shown in Supplemental Table 3. GBSS was cloned first into the pGEM-T Easy vector (Promega) and then into the pQE-80L expression vector (Qiagen), which incorporates a 6xHis amino tag [Nt-(His)6GBSS], and transformed into the Escherichia coli BL21 strain. Ten milliliters of an overnight culture of BL21 carrying plasmid Nt-(His)6GBSS was added to 1 liter of Luria-Bertani medium containing 100 µg/mL ampicillin and grown at 30°C until the absorbance at 600 nm in a spectrophotometer (Shimadzu) reached 0.8 OD. Recombinant gene expression was induced by the addition of 1 mM isopropyl-β-D-1-thiogalactopyranoside and incubation during 4 h at 30°C, when cells were harvested by centrifugation. The pellet was suspended in lysis buffer (50 mM phosphate buffer, pH 8.0, 150 mM KCl, 1 mM phenylmethylsulfonyl fluoride, and 5 mM MgCl2) and disrupted by sonication. The crude lysate was centrifuged at 40,000g for 30 min at 4°C, and the supernatant was used for one-step affinity purification employing HisTrap HP Columns (GE Healthcare), eluting bound proteins under native conditions by an imidazole gradient. The concentration of pure protein samples was measured by the dye binding method employing ovalbumin as a standard, and purity grade was confirmed by SDS-PAGE electrophoresis.

**Granule-Bound Starch Synthase Assay**

Starch granules from leaves (600 mg fresh weight) were purified following the Percoll method described by Delure et al. (1992). Starch granules were repeatedly washed with 50 mM HEPES, pH 7.5, and finally resuspended in 200 µL of 100 mM Tricine, pH 8.5, 25 mM potassium acetate, 10 mM DTT, 5 mM EDTA, and 10 mM ADP[1-14C]Glc (specific activity 7.4 GBq/mmol). After complete resuspension of starch granules, 100 µL was separated
and boiled for 5 min, representing time 0 of the assay. The rest of the reaction was incubated at 30°C for 1 h, and the reaction was stopped by boiling for 5 min. Starch granules were washed three times with a solution of 70% (v/v) methanol and 1% (w/v) KCl as described (Denyer et al., 1995), and radioactivity incorporated into the starch granules was determined with a scintillation counter.

**Purified Anti-GBSS Production**

Pure heterologous GBSS protein was used to produce polyclonal antibodies in rabbit against the Arabidopsis GBSS cDNA product without the signal peptide. Whole rabbit serum was used as a source of GBSS antibodies.

**Gel Electrophoresis and Immunoblots**

Proteins were separated by SDS-PAGE on 9% (w/v) polyacrylamide gels and either stained with Coomassie Brilliant Blue R 250 or transferred to nitrocellulose membranes employing a Trans-Blot SD transfer cell (Bio-Rad) according to the manufacturer’s instructions. Membranes were incubated in Tris-buffered saline containing 5% nonfat milk (w/v), and blots were sequentially probed with anti-GBSS serum followed by horseradish peroxidase–conjugated goat anti-rabbit serum. Blots were developed with a chemiluminescent substrate according to the manufacturer’s instructions (Immobilon Western Chemiluminescent HRP Substrate; Millipore).

**ChIP Protocol**

ChIP experiments were performed employing nuclear extracts (Lázaro et al., 2012) obtained from transgenic plants expressing CO-TAP-TAG fusion protein (CTAPI; Rohila et al., 2004), following the protocol of Haring et al. (2007).

**Microscopy Techniques**

**Plant Recombinant Protein Fusions**

Complete Arabidopsis GBSS ORF (1833 bp, including transit peptide) was amplified from a cDNA plasmid employing Gateway-designed primers (Invitrogen) and cloned into the pDONR207 vector. This plasmid was recombined into vector pEarleyGate100 (Earley et al., 2006) to obtain the final P3SS:GBSS construct and transformed in gbs mutant plants. To express the GBSS:GFP fusion under the GBSS promoter, the fragment 1 kb upstream of the predicted transcriptional start was cloned upstream of the GBSS ORF in vector pGW04 (Nakagawa et al., 2007) employing a two-step Gateway strategy. Recombinant plants expressing fluorescent proteins were observed by confocal microscopy (Leica TCS SP2) with 488-nm excitation light for GFP. The fluorescent signal was observed at 500 to 520 nm.

**Accession Numbers**

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: GBSS, AT1G32900; CO, AT5G1584; FT, AT1G65480; SEX1, AT1G10760; APS1, AT5G48300; SOC1, AT2G45360; GL, AT1G22770; CCA1, AT2G46830; LHY, AT1G01060; CDP1, AT2G32950; HOS1, AT2G39810; CDF1, AT5G23040; and UBQ10, AT4G05320.

**Supplemental Data**

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

**Supplemental Figure 1.** Changes in Starch and Soluble Sugar Contents in Col-0 and Ler Ecotypes during the Floral Transition.

**Supplemental Figure 2.** Purification of Recombinant GBSS, Activity, and Protein Levels in Different Recombinant Plant Lines and Complementation Analysis of the gbs Mutant.

**Supplemental Figure 3.** Starch Composition and Sugar Content in Starch-Less and Starch Excess Mutants and Their Flowering Time Phenotype.

**Supplemental Figure 4.** Gene Coexpression Analysis.

**Supplemental Figure 5.** Presence of GBSS in Different Conditions and Plant Lines.

**Supplemental Figure 6.** Distribution of GBSS:GFP Fusion Protein in GBSS:GBSS:GFP (gbs-1) Plants Resembles Wild-Type GBSS Distribution.

**Supplemental Figure 7.** Flowering Phenotype of gbs Mutants.

**Supplemental Figure 8.** Changes in CO Expression Alter FT mRNA Levels and Starch Accumulation.

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

**Supplemental Table 1.** GO Categories Significantly Enriched in the Different Modules Identified in the Gene Coexpression Network.

**Supplemental Table 2.** Flowering Time in LD and SD of Wild-Type, Mutant, and Recombinant Plants.

**Supplemental Table 3.** List of Primers Used in This Work.

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**AUTHOR CONTRIBUTIONS**

M.I.-M. and T.A. codesigned 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 and designed the experimental work and wrote the article.

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**REFERENCES**


