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Photoperiodic control of sugar release during the floral transition: What is the role of sugars in the florigenic signal?

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Florigen is a mobile signal released by the leaves that reaching the shoot apical meristem (SAM), changes its developmental program from vegetative to reproductive. The protein FLOWERING LOCUS T (FT) constitutes an important element of the florigen, but other components such as sugars, have been also proposed to be part of this signal.[1-5] We have studied the accumulation and composition of starch during the floral transition in *Arabidopsis thaliana* in order to understand the role of carbon mobilization in this process. In *A. thaliana* and *Antirrhinum majus* the gene coding for the Granule-Bound Starch Synthase (*GBSS*) is regulated by the circadian clock[6,7] while in the green alga *Chlamydomonas reinhardtii* the homolog gene *CrGBSS* is controlled by photoperiod and circadian signals.[8,9] In a recent paper[10] we described the role of the central photoperiodic factor CONSTANS (CO) in the regulation of *GBSS* expression in *Arabidopsis*. This regulation is in the basis of the change in the balance between starch and free sugars observed during the floral transition. We propose that this regulation may contribute to the florigenic signal and to the increase in sugar transport required during the flowering process.

Introduction

Plant life cycle is influenced by environmental conditions that affect their ability to obtain energy for its correct growth and development.[11] The floral transition is a crucial decision in the life cycle of a plant, so that failing to trigger the reproductive signal at the right time of the year, has a serious impact on the ability to produce offspring. For this reason, the process is tightly regulated[12] and plants have solved the problem by synchronizing their life cycle to the changing seasons. This timing is particularly important in intermediate latitudes where changes in environmental conditions are marked and predictable throughout the year. The photoperiod pathway controls the floral transition in response to day length in *Arabidopsis thaliana* and *CO* is a central gene in this process.[13] The involvement of *CO* in regulating the photoperiod response is evolutionarily conserved.[8,14] Thus, *CrCO*, a *CO* ortholog, controls the photoperiodic response in the green alga *C. reinhardtii* and directly affects starch metabolism through the transcriptional control of the algal ortholog of the *GBSS* gene.[8] Starch is the most important form of carbon reserve in plants. The starch granule contains 2 types of polymer, branched amylopectin, synthesized by Soluble Starch Synthases (SSS) and Starch Branching Enzyme (SBE); and linear amylose synthesized exclusively by GBSS.[15] Here we have focused our studies on transitory starch in *Arabidopsis* that is synthesized during the day and partially degraded during the night.[16]

We have recently described that the same photoperiodic signal that activates the expression of *FT* through CO is also responsible for the mobilization of sugars during the floral transition.[10] This action is mediated by GBSS. GBSS therefore, has a great influence on the composition of starch granule and its structure, in addition to the ability of plants to accumulate and mobilize sugars from it.[15] Therefore, the regulation and modification of *GBSS* expression levels could have an effect on the composition of the starch granule and the floral transition. In this paper we will further discuss the role of the photoperiodic signaling in the control of sugar release in *Arabidopsis* through the conserved control of *GBSS* expression and the significance of the sugar burst during the induction of flowering.

Results and Discussion

Starch and free sugar accumulation depends on the length of the day

We have described a mechanism involved in starch mobilization mediated by photoperiodic signals in long day (LD: 16h light/8h dark) in different *Arabidopsis* wild type ecotypes.[10] We

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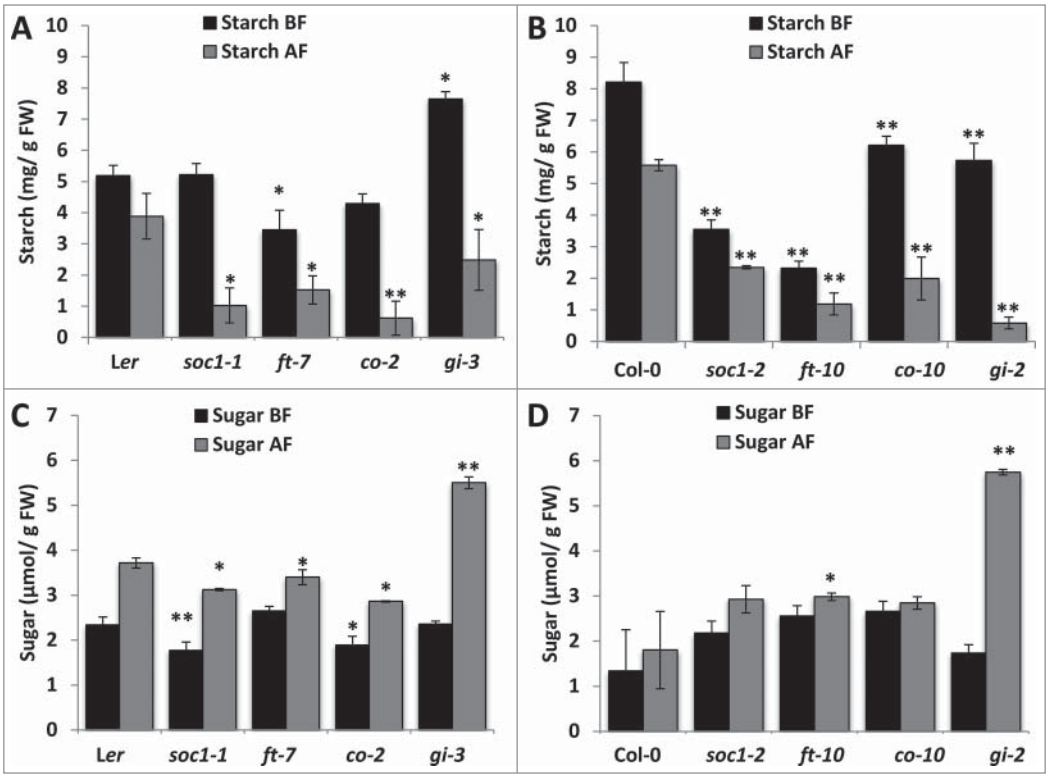

Figure 1. Starch and whole sugar content in Col-0 and L*er* mutants BF and AF. (**A**) Starch content in L*er* plants and mutant lines *soc1-1, ft-7, co-2 and gi-3*. (**B**) Starch content in Col-0 plants and mutant lines *soc1-2, ft-10, co-10 and gi-2*. (**C**) Level of main soluble sugars (glucose, fructose and sucrose) in the same plants as in A. (**D**) Level of main soluble sugars (glucose, fructose and sucrose) in the same plants as in B. Samples were collected in LD at the end of the day period before (black, BF) and after (gray, AF) flowering. Data represent the means of 3 biological replicates ± s.e.m. Significant differences (Student *t*-test) between WT BF and AF are marked by asterisk $*P < 0.01$ and $**P < 0.001$.

now show that starch and sugar content are particularly modified in mutant lines of the photoperiodic pathway (**Fig. 1**).[17] In L*er* ecotype background (**Fig. 1A**) starch accumulation was significantly reduced after flowering (AF) in all photoperiodic mutant lines analyzed. The proportion in starch level reduction AF in *gi-3* was lower compared to other photoperiod mutants, which may be due to the fact that gi-3 mutant also shows higher levels of starch before flowering (BF). This may be related to the fact that GIGANTEA (GI) is also involved in regulating carbohydrate accumulation through the circadian clock.[18-20] Interestingly, all photoperiod mutants analyzed in Col-0 background (**Fig. 1B**) showed a significant reduction in starch accumulation both BF and AF compared to wild type plants. This may reflect differences in the photoperiod response in relation to sugar mobilization between both ecotypes.

We also analyzed free sugar levels in L*er* and Col-0 mutants of the photoperiod pathway BF and AF (**Fig. 1C and D**). Mutants of both ecotypes showed some differences related to WT plants, but they were less obvious than differences in starch levels. As with starch accumulation, photoperiod mutants in L*er* and Col-0 backgrounds exhibited a differential free sugar accumulation pattern. Again, this was particularly true for *gi* mutants that showed very high levels of free sugars AF in both ecotypes. It should be noted that these measurements were performed at ZT16, time that corresponds to the maximum level of starch accumulation, while we had demonstrated that the most marked differences in sugar levels take place at ZT8.[10] Nevertheless, this analysis indicates that during the floral transition a difference in the accumulation of free sugars takes place and that it may also change depending on the photoperiod. Since mutant plants in the photoperiod pathway genes are particularly affected in their ability to accumulate starch and free sugars during the floral transition, the day length signals must have an important role in this accumulation.

Amylose levels are directly related with flowering time

Transitory starch synthesis and degradation, as well as *GBSS* mRNA levels, are under circadian regulation both in higher plants and algae.[6,21-22] We have also shown that starch accumulation, amylose synthesis and endogenous levels of sugars in *Arabidopsis* leaves vary drastically before and after the floral transition.[10] The mobilization of sugars from amylose allows plants to carry out successfully the transition from vegetative to reproductive state. In this sense, it was interesting to study plants with abnormal internal sugar levels in order to confirm this hypothesis.

For this reason we isolated T-DNA insertion mutants in the *GBSS* gene in *Arabidopsis* and studied their capacity to accumulate starch and sugars.[10] *gbs* mutants were unable to synthesize amylose and accumulated lower levels of sugars during the daytime. In addition, they exhibited a significant delay in flowering time. The linear composition of amylose may constitute an effective carbohydrate reserve that could be readily used to augment the levels of cellular free sugars during the floral transition. In this way, the lack of amylose would alter the capacity to release sugars and would thus produce a delay in the floral transition. We have observed this behavior in our studies on *gbs* mutants and other mutants related with starch composition and storage.

A similar flowering phenotype is observed in mutants affected in starch metabolism such as *aps1* (a starchless mutant)[23] and

sex1 (unable to degrade starch).[24] Both present a late flowering phenotype in LD. *sex1* mutant showed continuous high levels of starch during 24 h cycles and levels of free sugars were constantly lower than wild type plants over time.[10] On the other hand, *aps1* mutant showed the opposite effect, it did not present detectable levels of starch and sugars were constantly higher than in Col-0 plants.[10] Previous results also showed that *sex1* contained high levels of amylose,[24] so the low levels of free sugars observed in this mutant suggests that *sex1* is unable to mobilize sugars from this polymer during the transition to flowering. In summary, all mutants affected in starch metabolism studied (*gbs*, *aps1* and *sex1*) shared a common characteristic: they were unable to mobilize sugars from amylose. These results suggest that amylose is essential for the release of sugars during the floral transition. Consequently, a change in amylose accumulation is traduced into changes in flowering time.

CO induces *GBSS* expression in a photoperiod-dependent manner

The flowering delay in LD of the *gbs* mutants and its day length-dependent expression, suggested that the photoperiodic pathway could be controlling *GBSS* transcription and that CO could be involved in this process. Further confirmation came from the effect of *CO* mutation and overexpression on *GBSS* expression, so that *co-10* mutant showed a specific decrease in *GBSS* expression in the morning, while 35S:*CO* plants showed a marked increase of *GBSS* mRNA levels in the morning and a new expression peak in the evening.[10] *GBSS* expression pattern in short day (SD: 8h light/16h dark) did not show any modification in *CO* mutant and overexpressor lines and no delay in flowering time in SD of these lines was observed. As CO is not active in SD, this is precisely what was expected from a true CO target. In fact, using ChiP experiments, we also demonstrated that CO could bind directly to *GBSS* promoter in similar binding sites than those described for the *FT* promoter and this binding differed BF and AF.[10]

We have here confirmed that GBSS acts through CO by crossing *gbs* mutant into 35S:*CO* plants. The inclusion of *gbs* mutation in the overexpressing background delayed flowering time (**Fig. 2A**). We further demonstrate that this delay in flowering time is due to a reduced *FT* expression (**Fig. 2B**). Thus, CO capacity to alter *FT* expression is reduced in an amylose-free plant with reduced capacity to mobilize sugars during the floral transition.

Nevertheless, the delay in flowering time observed in the *co-10 gbs-1* double mutant in LD (**Fig. 2A**) indicates that in a very late flowering plant, GBSS must have a developmental role independently of CO. In fact, a double *gi-2 gbs1* mutant plant also flowered later than any of the progenitors (data not shown).

GBSS fusion to GFP reports starch presence in diverse locations and developmental stages

We have produced plants that express a translational fusion of GFP to the carboxyl part of GBSS driven by 1 kb from the *GBSS* promoter (*pGBSS*:*GBSS*:GFP).[10] Different tissue samples from recombinant *Arabidopsis* plants have been observed in

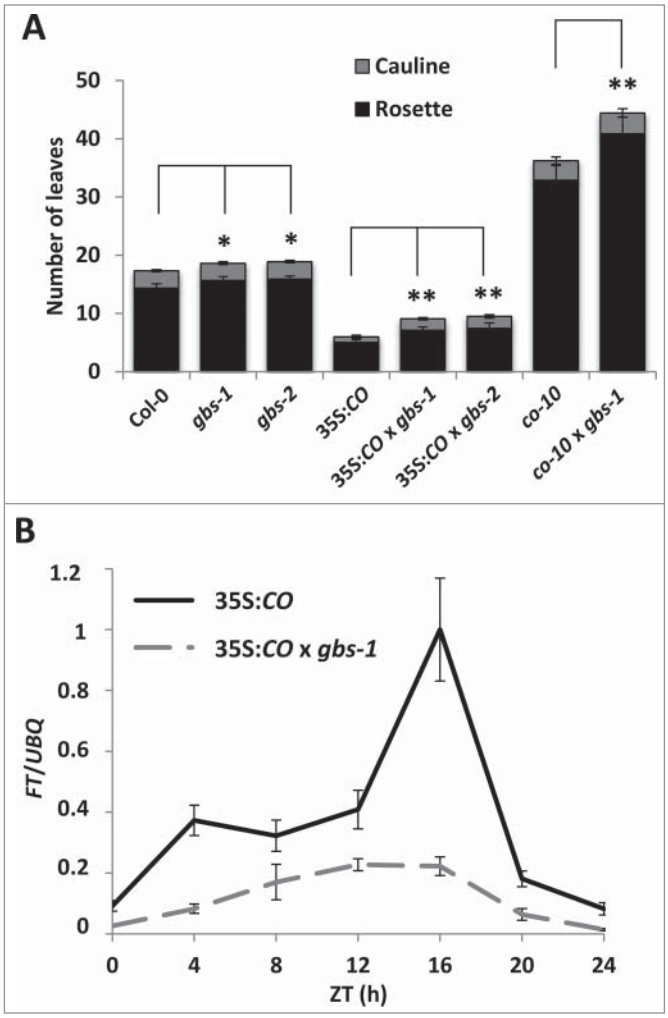

Figure 2. Effect of *gbs* mutation on *CO* overexpressing and mutant lines. (**A**) Number of leaves (rosette black, cauline gray) at the moment of flowering of Col-0, *gbs-1*, *gbs-2*, 35S:*CO*, 35S:*CO* x *gbs-1*, 35S:*CO* x *gbs-2*, *co-10* and *co-10* x *gbs-1* in LD. Data represent the means of 3 biological replicates ± s.e.m. Significant differences (Student *t*-test) between parental line BF and AF are marked by asterisk $*P < 0.01$ and $**P < 0.001$. (**B**) Q-PCR analysis of *FT* expression in 35S:*CO* (black) and 35S:*CO* x *gbs-1* (gray) plants during a 24-h LD BF experiment.

diverse developmental stages under the confocal microscope (**Fig. 3**). GFP was detected in chloroplasts from all green tissues, but in some cases we noticed that GBSS presence, and thus starch accumulation, was present at a different developmental stage than previously suggested.

Developing seeds showed a clear GBSS:GFP signal (**Fig. 3A–C**). In early silique developmental stages, GFP signal was uniformly distributed in the seed (**Fig. 3A and B**), whereas in later developmental stages, GBSS was restricted to the outer seed region that can be attributed to the aleurone cell layer. In these mature seeds, although most of carbohydrates have been already converted into reserve fatty acids,[25] the aleurone cell layer remains clearly visible. This suggests that the presence of starch in *Arabidopsis* seeds, probably feeding sugars to the developed

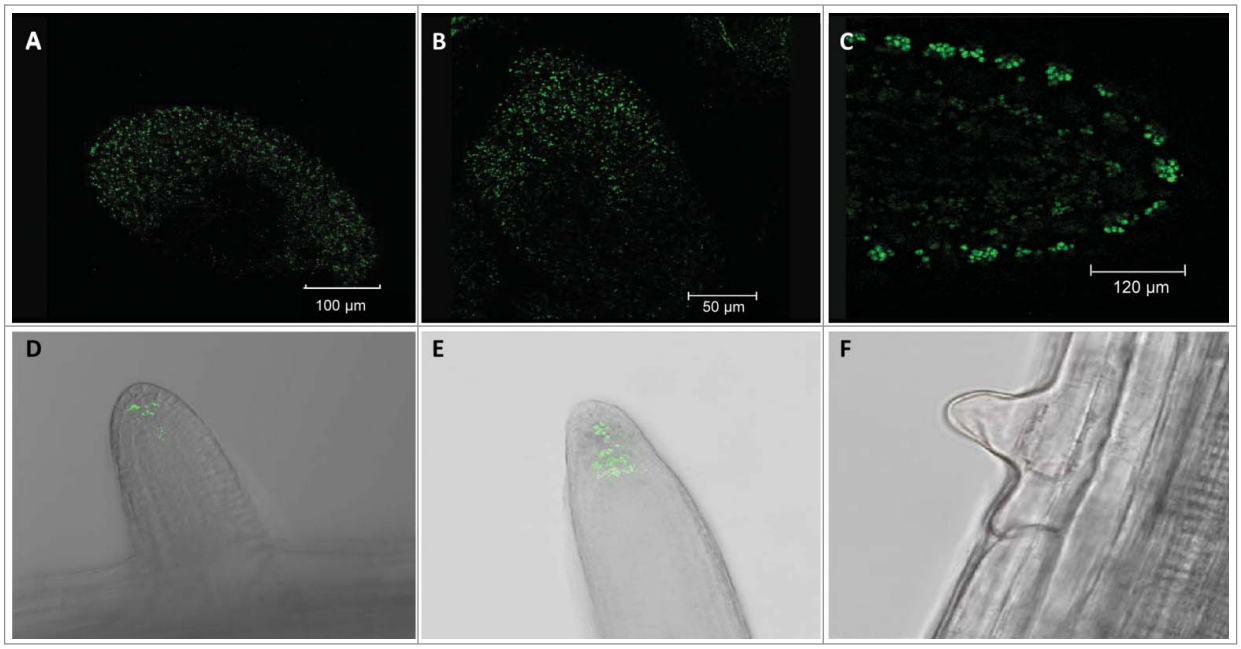

Figure 3. Presence of GBSS protein *in vivo* in different stages of seed (**A–C**) and lateral root development (**D–F**). *pGBSS*:*GBSS*:GFP plants were grown in LD and GFP presence was observed under the confocal microscope. Upper panel: GBSS presence in seeds in 3 different developmental stages (**A**) early seed, (**B**) medium stage seed and (**C**) mature seed. GFP fluorescence is associated to the aleurone cell layer in mature seeds. Lower panel: GBSS presence in root organs in different developmental stages: (**D**) early lateral root formation, (**E**) late stage lateral root formation, (**F**) root hairs. GBSS is progressively associated to the columella cell layer of the lateral root meristem but is absent from root hairs.

embryo, remains active longer than previously reported.[26] It will be of interest to determine whether this accumulated starch in mature seeds has a significant role during germination.

In the root meristem, GBSS was detected in the columella cell layers where starch accumulates conferring, among other physiological functions, a gravitropic response to the growing root. We have further detected GBSS:GFP fusion in the growing lateral roots, accounting for the observed gravitropic response of these lateral organs (**Fig. 3D and F**).[27] As expected, we could not detect GFP presence in root hairs (**Fig 3G**). Thus, GBSS and starch seem to be restricted to root organs responding to gravity, although their involvement in other physiological processes can not be ruled out.

Conclusions. Florigen is a complex signal probably composed of several different elements and, although mainly of a photoperiodic nature, may also be influenced by other environmental cues. Sugars are not only a source for structural and energetic physiological processes, but can also have an important signaling role.[1,5] Here, we describe how the same mechanism that controls the production of FT is also used by the plant to alter starch composition in order to facilitate a coordinated release of sugars. A possible physiological function for the increase in sugar levels exactly during the floral transition may be related to a role as osmotic pull for the florigen transport through the phloem. Nevertheless, our studies cannot rule out the possibility that this sugar burst during the floral transition could also be used as a reinforcement signal to that triggered by FT or that it constitutes *per se* an independent sugar-based signal that is part of the florigen. Therefore, we conclude that the effect of *CO* on *GBSS* could be one of the mechanisms involved in coordinating the induction of flowering by photoperiod and carbon mobilization. Further studies on the nature of these signals and the role in activating downstream genes involved in the floral meristem identity will be needed to support these hypotheses.

Methods

Plant Material and Growth Conditions

Plants were grown in controlled cabinets on peat-based compost under long day conditions (LD: 16h light/8h dark) as described in Ortiz-Marchena et al.[10] Leaf samples were taken 2 d before and after flowering induction. *Arabidopsis thaliana* plants including wild type Col-0 and L*er, gbs-1*, *gbs-2,* 35S:*CO* x *gbs-1* and 35S:*CO* x *gbs-2* have been decribed in Ortiz-Marchena et al.[10] The *aps1* mutant was reported by Ventriglia et al.[23] The *sex-1* mutant was reported by Yu et al.[24] The *co-10* mutant was reported by Laubinger et al.[28] The *co-2* mutant was reported by Simon et al.[29] The *ft-10* mutant was reported by Jang et al.[30] The *ft-7* mutant was reported by Onouchi et al.[31] The *soc1-2* mutant was reported by Lee et al.[32] The *gi-3* and *gi-2* mutants were reported by Fowler et al.[18] Recombinant plant expressing fluorescent protein GBSS:GFP was decribed by Ortiz-Marchena et al.[10]

Starch Analysis

Starch granules were extracted by a modification of the method described by Huber[33] as described in Ortiz-Marchena et al.[10] and Albi et al.[34]

Determination of Soluble Sugars

Whole content of main soluble sugars (glucose, fructose and sucrose) were identified and quantified employing a high-performance anion-exchange chromatography protocol as described in Ortiz-Marchena et al.[10, 35]

Real-Time Q-PCR

Q-PCR was performed in an iQTM5 multicolor real-time PCR detection system from Bio-Rad as described in Ortiz-Marchena et al.[10] Normalized data were calculated by dividing the average of at least 3 replicates of each sample from the candidate and reference genes. Primers for QPCR mRNA amplification for *FT*: 5′-CGAACGGTGATGATGCCTATAGTAG-3′ and 5′-CACTCTCATTTTCCTCCCCCTCTC-3′ *and UBIQ10* 5′-GAAGTTCAATGTTTCGTTTCATGT-3′ and 5′-GGATTATACAAGGCCCCAAAA-3′.

Confocal microscopy

Recombinant plants expressing fluorescent proteins were observed by confocal microscopy (Leica TCS SP2) as described in Ortiz-Marchena et al.[10]

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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