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Expression of the Granule-Bound Starch Synthase I (*Waxy*) Gene from Snapdragon Is Developmentally and Circadian Clock Regulated[1]

Angel Mérida, José M. Rodríguez-Galán[2], Coral Vincent, and José M. Romero*

Instituto de Bioquímica Vegetal y Fotosíntesis, Centro de Investigaciones Científicas Isla de la Cartuja, C/Americo Vespucio s/n, 41092 Seville, Spain (A.M., J.M.R.-G., J.M.R.); and John Innes Centre, Norwich NR4 7UH, United Kingdom (C.V.)

The granule-bound starch synthase I (GBSSI or waxy) enzyme catalyzes one of the enzymatic steps of starch synthesis. This enzyme is responsible for the synthesis of amylose and is also involved in building the final structure of amylopectin. Little is known about expression of GBSSI genes in tissues other than storage organs, such as seeds, endosperm, and tuber. We have isolated a gene encoding the GBSSI from snapdragon (*Antirrhinum majus*). This gene is present as a single copy in the snapdragon genome. There is a precise spatial and developmental regulation of its expression in flowers. *GBSSI* expression was observed in all floral whorls at early developmental stages, but it was restricted to carpel before anthesis. These results give new insights into the role of starch in later reproductive events such as seed filling. In leaves the mRNA level of *GBSSI* is regulated by an endogenous circadian clock, indicating that the transition from day to night may be accompanied by abolition of expression of starch synthesis genes. This mechanism does not operate in sink tissues such as roots when grown in the dark.

Starch is the most important form of C reserve in plants. Long-term storage starch is found in storage organs such as tubers, endosperm, embryos, or storage roots, and transitory starch is present in photosynthetically active tissues such as leaves. The starch biosynthesis pathway has been extensively studied in diverse species such as maize, potato, pea, and others (for review, see Martin and Smith, 1995; Nelson and Pan, 1995; Preiss and Sivak, 1996; Smith et al., 1997), and considerable progress has been made toward understanding the role of each enzymatic step needed to build the final structure of the starch granule (Ball et al., 1996; Buleon et al., 1997). These studies have demonstrated that plants use different isoforms for each enzyme of the biosynthetic pathway: ADP-Glc pyrophosphorylase, starch synthases, and starch-branching enzyme, and each isoform is encoded by a different gene. For example, three isoforms for starch-branching enzyme have been found in maize (Boyer and Preiss, 1981), and the genes encoding the three isoforms have been previously described (Baba et al., 1991; Fisher et al., 1995; Gao et al., 1997). Three genes encoding for the large subunit of the ADP-Glc pyrophosphorylase have been cloned in Arabidopsis (Villand et al., 1993). In potato three genes encoding distinct isoforms of the starch synthase have been previously described (Van der Steege et al., 1992; Edwards et al., 1995; Marshall et al., 1996; Abel et al., 1997).

One of these isoforms, GBSSI, is located exclusively in the starch granule (Sivak et al., 1993). It was originally identified in maize as the product of the *waxy* gene, and through mutant analysis it has been shown to be responsible for the synthesis of linear glucan (amylose) in starch (Nelson and Pan, 1995). Genes encoding the orthologous protein have been isolated from many different plant species such as potato (Van der Steege et al., 1992), pea (Dry et al., 1992), barley (Rohde et al., 1988), and wheat (Clark et al., 1991). Analysis of mutants in rice, Amaranthus, potato, and pea has demonstrated that *GBSSI* is also responsible for the synthesis of amylose in storage organs of these plants (Smith et al., 1997, and refs. therein). However, on the basis of biochemical and mutant studies, the existence of different isoforms responsible for the GBSSI activity in different organs has been proposed for pea (Denyer et al., 1997), rice (Taira et al., 1991), and wheat (Nakamura et al., 1998).

Most of the studies of starch biosynthesis have been carried out on storage organs such as tuber or endosperm because of the economic importance of the long-term reserve form of starch and the relative availability of both enzymes and product. There is much less information available about starch synthesis in other organs and tissues of the plant, although changes in the synthesis and mobilization of transitory starch affect processes such as growth rate, flowering time, and seed filling (Bernier et al., 1993; Schulze et al., 1994). It has been proposed that mobilization of starch stored in leaves and stems to Suc provides one of the early signals for the induction of flowering (Bernier et al., 1993). Analysis of the growth of a starch-less mutant of

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[2] Present address: Instituto de Recursos Naturales y Agrobiología. Avda Reina Mercedes no. 10, 41012-Sevilla, Spain.

* Corresponding author; e-mail jmromero@cica.es; fax 34–54–46–0065.

Abbreviations: GBSSI, granule-bound starch synthase I; RT, reverse-transcriptase.

Arabidopsis (deficient in plastidial phosphoglucomutase activity; Caspar et al., 1985) has shown that synthesis of starch is necessary not only to maintain normal growth rates under a natural day/night regime but also to promote other developmental changes such as flowering or seed filling (Schulze et al., 1994).

The main characteristic of transitory starch is that it is synthesized during the day and degraded during the night to supply the C requirements of the plant. A circadian regulation of starch accumulation during the day has been proposed (Li et al., 1992; Geiger and Servaites, 1994). However, little is known about the activity of starch biosynthetic enzymes or the expression levels of their genes during the day/night cycle. Thus, the point at which circadian regulation of starch synthesis might operate remains unclear.

In this study we cloned and characterized a gene encoding the GBSSI protein. We show that in snapdragon (*Antirrhinum majus*) this gene is present as a single copy and is responsible for GBSSI activity in all organs and tissues in which amylose is synthesized. A detailed study of the expression of this gene has been performed. Results show high expression in meristematic tissues and precise spatial and developmental regulation of its expression during flower development. We also show a circadian regulation of *GBSSI* transcript levels in leaves. *GBSSI* mRNA levels decrease at the end of the day and cannot be detected during the night even when plants are maintained under constant illumination. Sequence comparison of the promoter region of the *GBSSI* gene with the promoters of other genes regulated by the circadian clock identify the presence of conserved *cis* elements that have been shown to be critical components of this regulation. The integration of regulation of starch synthesis into a more general circadian regulation of C metabolism is discussed.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Snapdragon (*Antirrhinum majus* stock JI:7, John Innes Centre, Norwich, UK) was grown in a greenhouse under a natural day/night regime. Plants cultured in growth cabinets were grown under a 16-h light/8-h dark photoregime at 23°C (day)/20°C (night), 70% RH, and a light intensity at the plant level of 120 μE m^{-2} s^{-1} supplied by white fluorescent lamps.

Isolation of a cDNA Fragment Encoding GBSSI

A cDNA library made with mRNA from snapdragon leaves in λgt10 was screened with a cDNA fragment of the pea *GBSSI* gene (Dry et al., 1992). Filters were hybridized in 6× SSC, 0.1% SDS, 0.02% Ficoll, 0.02% PVP, and 5 μg/mL salmon sperm DNA at 55°C and washed in 2× SSC and 0.5% SDS at 55°C. From eight positive clones, the one with the largest insert (2.3 kb) was selected, and the cDNA fragment was cloned into the pBluescript II SK(−) plasmid.

Isolation of Genomic Clones

A snapdragon genomic library in λEMBL4 was screened with the 2.3-kb cDNA fragment previously cloned. Filters were hybridized as described for the cDNA library at 55°C and washed in 0.1× SSC and 1% SDS. Twelve positive clones were obtained and showed overlapping fragments by restriction analysis.

Sequencing

Sequences were determined according to the method of Sanger et al. (1977) by double-stranded plasmid sequencing in pBluescript using the T7-sequencing kit (Pharmacia Biotech).

Extraction and Gel Analysis of DNA and RNA

DNA was extracted from leaves of snapdragon according to the method of Martin et al. (1985). Southern hybridizations were performed as described by Sommer et al. (1985). Total RNA was extracted as described previously by Prescott and Martin (1987) and was analyzed by electrophoresis through formaldehyde gels and transferred to GeneScreen Plus filters (NEN Life Science Products). Filter hybridizations were performed according to the manufacturer's instructions.

RT-PCR Amplification

cDNA was amplified by PCR according to the method of Frohman et al. (1989). First-strand cDNA was synthesized from 10 μg of total RNA using the (dT)-17 adaptor as the primer. To stop the reaction the sample was diluted to 1 mL with water. A 10-μL sample was taken for amplification using oligonucleotides from different regions of the *GBSSI* gene. Amplification involved 40 or 20 cycles with a denaturation time of 1 min at 94°C, an annealing time of 90 s at 55°C, and an extension time of 3 min at 72°C.

In Situ Hybridization Analysis

In situ hybridization was performed as described by Bradley et al. (1993). Digoxigenin labeling of RNA and detection of signal were performed using Boehringer Mannheim methods and reagents (kit no. 1175-041) with some modifications as described previously by Coen et al. (1990). Plasmids pSA301 and pSA302, containing 1 and 0.5 kb encoding for amino acids 1 to 367 and 367 to 547, respectively, were cut at the 5′ end of the insert and transcribed in the presence of digoxigenin-labeled nucleotides. The same plasmids cut at the 3′ end were used to transcribe the sense probes.

The resulting probes were hydrolyzed to generate fragments of about 150 bp, and sections of different tissues were probed. After the hybridization signal was detected, sections were counterstained with Fluorescent Brightener 28 (Sigma) and mounted in Entellan mounting medium (Merck, Darmstadt, Germany). Sections were photo-

graphed through a Nikon Microphot-SA microscope using Kodak ASA 200 Ektachrome film.

Starch Measurement

Leaf starch was quantified as described previously by Lin et al. (1988). Starch was converted to Glc by incubation with amyloglucosidase (from *Aspergillus niger*, Sigma), and Glc amount was analyzed enzymatically using hexokinase and Glc-6-P dehydrogenase (Jones et al., 1977).

Iodine Staining of Starch Granules

Tissue sections (7 μm) fixed and wax embedded, as described by Bradley et al. (1993), were treated with xylene to remove the wax and then stained with Lugol solution. Stained slides were rinsed with distilled water, and starch granules were visualized using a zoom stereomicroscope (model SZ4045TR, Olympus). Images were captured with a color video camera (model JVC TK-C1381), using the MicroImage image analysis software (Olympus).

RESULTS

Isolation of cDNA and Genomic Fragments Encoding for GBSSI

A cDNA fragment encoding for the GBSSI from pea (Dry et al., 1992) was used to screen a λgt10 cDNA library made from mRNA from snapdragon leaves. Eight positive clones were isolated, and the clone containing the largest cDNA fragment (2.3 kb) was selected for sequencing. This fragment contains an open reading frame encoding a 608-amino acid polypeptide with a predicted molecular mass of 66,360 D. The deduced amino acid sequence is very similar to other GBSSI proteins previously described: 84%, 75%, and 71% of amino acid identity to GBSSI from potato, pea, and barley, respectively (Rohde et al., 1988; Dry et al., 1992; Van der Steege et al., 1992), indicating that the cDNA selected encodes a snapdragon GBSSI protein.

The 2.3-kb cDNA fragment was used to screen a λEMBL4 snapdragon genomic library, and 12 positive clones were isolated. Those clones contained overlapping fragments as shown by restriction analysis (data not shown). By Southern analysis a 5-kb *Eco*RI genomic fragment was selected and subcloned into pBluescript II SK(−). This fragment contained the *GBSSI* gene and part of its promoter region.

GBSSI Is Present in a Single Copy in Snapdragon

The possible presence of more than one gene encoding for GBSSI in snapdragon was tested by Southern analysis. Genomic DNA was cut with different restriction enzymes and hybridized with the 2.3-kb cDNA fragment under low-stringency conditions (50°C hybridization temperature and 2× SSC and 0.1% SDS washing buffer). Results obtained are shown in Figure 1. Both *Eco*RI and *Xba*I digestions gave single hybridizing bands. *Bst*XI and the double-digestion *Bst*XI/*Xba*I rendered in both cases an intense band and a very faint one. These less intense bands correspond to hybridization with the 130 bp of the cDNA fragment used as a probe that extends upstream of the *Bst*XI site (see map of the region in Fig. 1). These results indicate that the *GBSSI* gene is present in a single copy in snapdragon and would be responsible for the granule-bound starch synthase activity in all of the organs of the plant.

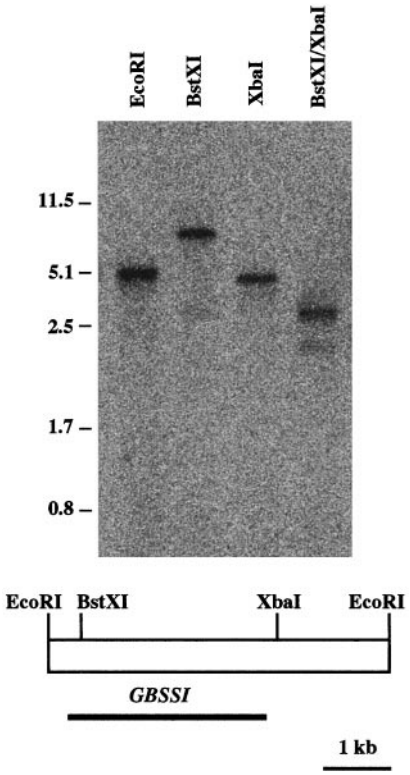

Figure 1. Restriction analysis of snapdragon genomic DNA. Twenty micrograms of genomic DNA was cut with *Eco*RI, *Bst*XI, *Xba*I, and *Bst*XI plus *Xba*I and analyzed by Southern analysis using the 2.3-kb full-length *GBSSI* cDNA fragment as a probe. The map at the bottom shows the cloned *Eco*RI genomic fragment and the position for *Bst*XI and *Xba*I sites. The region covered by the cDNA probe is indicated by a thick black bar.

Expression Pattern of the *GBSSI* Gene

We first studied the expression of the *GBSSI* gene in snapdragon by northern analysis. Figure 2A shows that the gene exhibits its highest level of expression in leaves and is expressed to an equal level in inflorescences (0.5 cm long), mature flowers, and fruits 7 d after pollination. Expression of the *GBSSI* gene in these organs was corroborated by RT-PCR using an oligonucleotide from the 3′ end of the gene and another from the 3′-untranslated region (Fig. 2B). These results clearly indicate that *GBSSI* is expressed in all organs studied. Expression in roots was extremely low and could be detected only by RT-PCR (Fig. 2B).

A more detailed study of the *GBSSI* expression pattern was performed by in situ mRNA hybridization. We used two different cDNA fragments to obtain probes, one fragment of 1 kb comprising the region encoding the first 367 amino acids and a 0.5-kb fragment containing the sequence

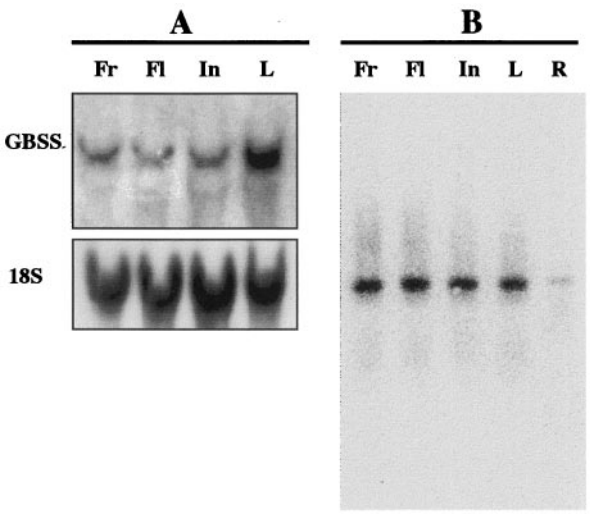

Figure 2. Analysis of *GBSSI* expression in different tissues. A, Northern analysis. Total RNA (30 μg) from fruits (lane Fr), flowers (lane Fl), 0.5-cm-long inflorescences (lane In), and leaves (lane L) was probed with the 2.3-kb *GBSSI* cDNA fragment. Loading was controlled by reprobing the filter with a cDNA fragment encoding the 18S rRNA from sunflower. Transcript in root samples was undetectable and is not shown. B, RT-PCR analysis of *GBSSI* transcript. Total RNA (10 μg) from fruits (lane Fr), flowers (lane Fl), 0.5-cm-long inflorescences (lane In), leaves (lane L), and roots (lane R) was used for single-strand cDNA synthesis and subsequent amplification by PCR (40 cycles) using oligonucleotides SA9 (955–936 bp upstream from the stop codon) and SA6 (65–45 bp downstream from the stop codon). PCR products were analyzed by Southern analysis using the whole *GBSSI* cDNA fragment as a probe. Loading in lane R was 10-fold higher than in the rest of the lanes to compensate for the low amplification obtained in this tissue.

encoding the amino acids 367 to 547 of GBSSI. In both cases we obtained the same pattern of expression in all of the tissues and organs studied. These results are consistent with those shown above, which show that *GBSSI* is encoded by a single-copy gene in snapdragon.

Expression in leaves was located mainly in the palisade parenchyma (Fig. 3A), being much lower or absent in the spongy cells of the mesophyll and suggesting that transient starch accumulation in leaves along the light period takes place almost exclusively in the palisade cells. This idea was supported by iodine staining of cross-sections of leaves, which showed accumulation of starch granules in the palisade parenchyma but not in the spongy cells (data not shown). *GBSSI* was also expressed in the parenchymatic tissue of the stem, located mainly in the subepidermal layer of cells (Fig. 3B). It is interesting that the expression of *GBSSI* was very intense in meristematic tissues, in both the apical meristem and in floral primordia (Fig. 3, C and D). High expression of *GBSSI* was detected in all three layers of the apical meristem (Fig. 3E). Iodine staining of these meristematic tissues showed starch accumulation in all regions where *GBSSI* was expressed (data not shown). These results indicate that meristematic tissues are able to synthesize their own reserve of carbohydrate, which could contribute in a significant manner to maintaining the elevated growth rates of these tissues.

GBSSI was highly expressed in floral primordia before organ differentiation. This high expression was maintained in subsequent developmental stages and was clearly visible in the four whorls in flower buds (Fig. 3, F and G). However, prior to anthesis, expression of *GBSSI* was lost in sepals, petals, and stamens and was subsequently detected only in the carpel (Fig. 3, H–J). The expression in the carpel was maintained throughout flower development and was also visible in the capsule once the fruit was formed. At early developmental stages there was clear expression of *GBSSI* in anthers (Fig. 3K) that was completely abolished prior to anthesis (Fig. 3L). By contrast, expression in carpels and ovules was maintained throughout flower development (in Fig. 3, J, L, and M correspond to carpel, anther, and ovule, respectively, at the same developmental stage), indicating different requirements for starch synthesis in these two reproductive organs. In fruit *GBSSI* expression was also detected in seeds (Fig. 3N), indicating that snapdragon accumulates starch as a reserve material in the seed endosperm (data confirmed by iodine staining, data not shown).

The presence of starch in flowers before anthesis was determined by iodine staining of flower sections at the same developmental stage as those shown in Figure 3, H to J. Figure 4, A and C, shows the presence of starch granules in the carpel and the style but also in sepals and petals. These results could indicate that in sepals and petals there is a modified form of starch granule lacking amylose. However, the iodine-stained starch of sepals and petals did not show the red color characteristic of amylose-free starch (Kuipers et al., 1994). Thus, the starch in these organs at this stage might result from synthesis at earlier developmental stages. In accordance with the in situ results, starch granules were also detected in the carpel. It is worth noting the high accumulation of starch in the funiculus, connecting the ovule to the ovary wall, and in the nectary (Fig. 3B, arrows), a tissue where carbohydrate metabolism is very active.

Expression of GBSSI through the Day/Night Cycle

The main feature of starch synthesis in leaves is its fluctuating character throughout the day/night cycle. It is well established that starch is accumulated in chloroplasts during the light period and mobilized in the dark, supplying C to the plant when the photosynthetic CO_2 fixation is not operative. This behavior is illustrated in Figure 5A. Fifteen-day-old snapdragon plants were adapted to a 16-h light/8-h dark period for 1 month, and then two fully expanded leaves of the same node were collected from successive plants at 2-h intervals throughout the cycle. The content of starch was determined enzymatically as described in "Materials and Methods."

At the beginning of the light period, a 2- to 4-h lag interval was observed before net accumulation of starch was detected (Fig. 5A). During this phase there was a slight decrease in the level of starch relative to the amount present at the end of the dark period. After the initial lag, the rate of starch accumulation remained relatively steady until approximately 2 h before the onset of the dark, when

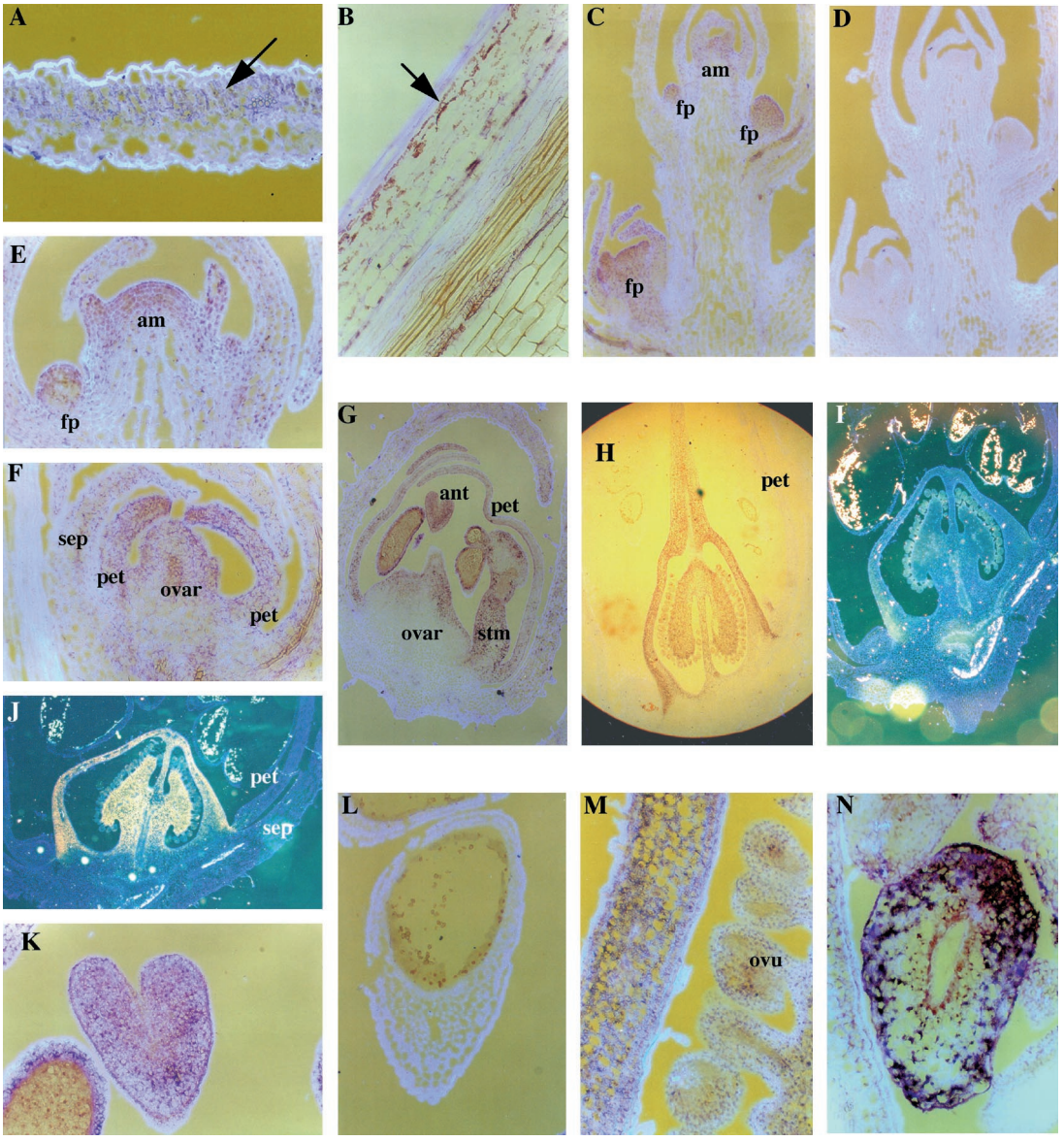

Figure 3. In situ hybridization of sections from different tissues with the digoxigenin-labeled *GBSSI* RNA probe. A, Transverse section of leaf. Arrow, Hybridization in the palisade parenchyma. B, Longitudinal section of stem. *GBSSI* expression in the subepidermal layer is indicated. C, Longitudinal section of a 0.5-cm-long inflorescence. Hybridization in apical meristem (am) and floral primordia (fp) is visible. D, Negative control of C using the sense probe. E, Longitudinal section of apical meristem. Expression in the three layers of the apical meristem (am), bracts, and floral primordia (fp) is apparent. F, Floral primordium once the whorls have been formed. Hybridization is visible in ovary (ovar), sepals (sep), and petals (pet). G, Longitudinal section of a flower bud. A clear expression in the four whorls, including stamen (stm) and anthers (ant), is shown. H, Longitudinal section of a flower before anthesis. *GBSSI* expression is restricted to the carpel. I and J, Flower at the same developmental stage as in H. Dark-field exposure was used to show the absence of expression in sepals (sep) and petals (pet) at this stage (J). I shows a negative control using the sense probe. K, Magnification showing a cross-section of an anther in a flower bud (same developmental stage as in G). L, Cross-section of anther before anthesis (same stage as H–J). No signal was detected at this developmental stage. M, Magnification of H showing *GBSSI* expression in ovules (ovu). N, Magnification of a longitudinal section of a capsule 7 d after pollination. Expression in the seed endosperm is clearly visible.

it began to decline. The starch accumulation rate had also declined by the end of the normal day even when the day period was extended (Fig. 5A, second cycle). During such extended light periods, the accumulation of starch stopped and starch levels remained steady with values oscillating at approximately 0.42 mg starch/g fresh weight. These results are in accordance with those reported by Li et al. (1992) for sugar beet leaves and suggest an endogenous circadian regulation of starch accumulation during the day/night cycle.

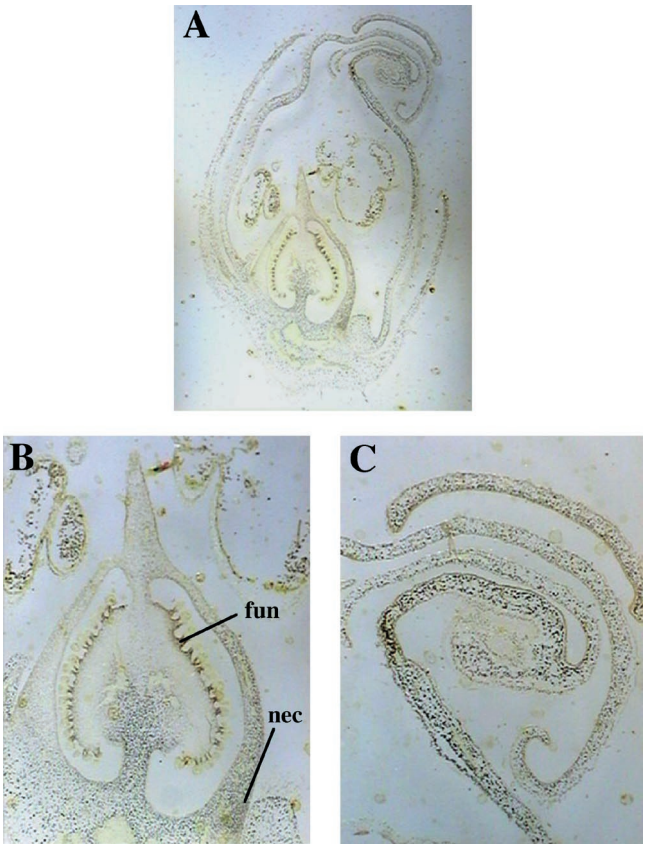

Figure 4. Iodine staining of longitudinal section of flowers before anthesis. Longitudinal sections of flowers at the same developmental stage as in Figure 3, H to J. Iodine staining was used to detect starch accumulation. A, Starch granule accumulation in the carpel, as well as petals and sepals. B and C, Magnification of A showing starch accumulation in carpel and petals, respectively. Accumulation of starch granules in the funiculus (fun) and in the nectary (nec) are indicated in B.

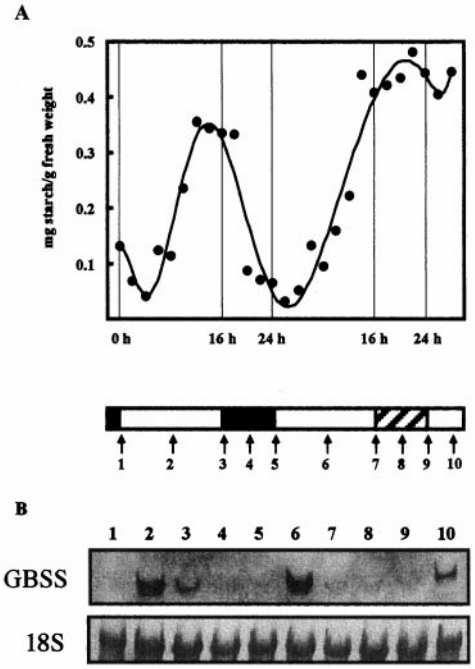

Figure 5. Starch accumulation and *GBSSI* expression in leaves during the day/night cycle. A, Starch accumulation in leaves during a 16-h light/8-h dark photoperiod regime. The bar at the bottom of the graph indicates the corresponding day/night periods: day (open box), night (closed box), and period corresponding to night but in which light was kept on (strippled box). B, Time course of *GBSSI* expression during a 16-h light/8-h dark photoregime**.** Arrows and numbers at the bottom of the bar indicate the time when samples were collected for RNA isolation. Loading was controlled by reprobing the filter with DNA encoding 18S RNA from sunflower. Starch content and *GBSSI* transcript levels in leaves were determined in three independent time-course experiments and the same results were obtained.

In the same time-course experiments, samples were collected for isolation of total RNA, and *GBSSI* mRNA levels during the cycle were analyzed by northern analysis. As shown in Figure 5B, the level of *GBSSI* mRNA was high at midday (Fig. 5B, lanes 2 and 6), was drastically reduced by the end of the day (Fig. 5B, lanes 3 and 7), and was almost undetectable during the night (Fig. 5B, lanes 1, 4, and 5). The *GBSSI* mRNA level was also severely reduced even when the light period was extended to the second night (Fig. 5B, lanes 8 and 9). Finally, 4 h after the end of the time corresponding to the second night period, the *GBSSI* transcript level was restored (Fig. 5B, lane 10). These results point to an endogenous circadian clock regulating *GBSSI* mRNA abundance, which, in turn, match the pattern of starch accumulation in leaves during the day/night cycle.

A light-entrained circadian control of *GBSSI* transcript levels would not be expected to occur in an organ such as the root. To test this hypothesis we compared the amounts of *GBSSI* mRNA in roots and leaves of plants collected at the midpoints in the day and night. Figure 6 shows the result of an RT-PCR (20 cycles of amplification) using RNA from leaves and roots. Although the presence of *GBSSI* mRNA could not be detected in leaves after 4 h of darkness (Fig. 5B), levels of *GBSSI* transcripts in roots remained unaffected. These results indicate that the mechanisms controlling *GBSSI* mRNA abundance in leaves during the day/night cycle do not operate in roots.

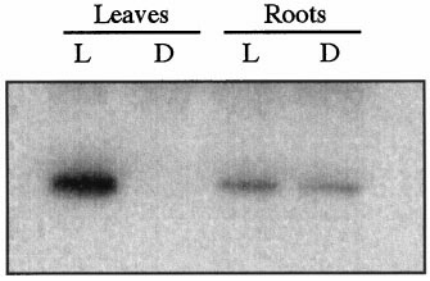

Figure 6. RT-PCR of *GBSSI* transcripts in leaves and roots during day and night. Samples were collected at the midpoint of the day (L) and night (D) periods. *GBSSI* transcripts amplified by PCR (20 cycles) were analyzed by Southern analysis. Loading in roots was 100 times greater than in leaves to compensate for the different levels of expression of *GBSSI* in these organs.

DISCUSSION

We have characterized the expression of the gene encoding GBSSI (or waxy protein) in snapdragon. It is well established that this enzyme is responsible for the synthesis of amylose in the starch granule, and mutants defective in this activity show an amylose-free starch (Kuipers et al., 1994; Denyer et al., 1995). A role for this enzyme has also been demonstrated in the synthesis of amylopectin, and mutants of *Chlamydomonas reinhardtii* lacking GBSSI activity have a structurally modified amylopectin (Delrue et al., 1992). Studies in several species have revealed the presence of distinct genes encoding different isoforms in all of the enzymatic steps of starch biosynthesis (Martin and Smith, 1995; Smith et al., 1997). In the case of the *GBSSI* gene, Hirano and coworkers showed that the *waxy* gene of rice is expressed in seeds and anthers exclusively (Hirano and Sano, 1991; Hirano et al., 1995), indicating that there are other isogenes responsible for the granule-bound starch synthase activity in other parts of the plant. In pea two isoforms of GBSSI have been detected that are differentially expressed in the plant (Denyer et al., 1997).

Unlike the situation in pea and rice, we detected expression of the *GBSSI* gene in all organs tested by northern blot hybridization: leaves, inflorescences, flowers, and fruits (Fig. 2A). RT-PCR analysis using an oligonucleotide of the 3′ end of the gene and another of the 3′-untranslated region confirmed the expression of *GBSSI* in those tissues, as well as in roots (Fig. 2B). Southern analysis of genomic DNA cut with different restriction enzymes clearly indicate that the *GBSSI* gene is present in a single copy in snapdragon and is responsible for all *GBSSI* transcripts.

Most of the studies of the expression of genes involved in starch biosynthesis have been carried out in storage organs. In this case, the levels of *GBSSI* mRNA peak later than other starch synthase isoforms during pea embryo development (Dry et al., 1992), and genes encoding for the distinct isoforms of the starch-branching enzyme are differentially expressed during the development of pea embryo (Burton et al., 1995) and wheat (Morell et al., 1997) and maize endosperm (Gao et al., 1997). However, there is very little information about the pattern of expression of the starch biosynthesis genes in tissues other than storage organs. In this report we have shown that *GBSSI* is highly expressed in the palisade parenchyma but is only weakly expressed in the spongy cells. This result correlates with data obtained by iodine staining of these sections, which showed that starch granules accumulate mainly in the palisade cells (data not shown) and indicate the dominant role of this tissue in the supply of C skeletons derived from starch to the rest of the plant. *GBSSI* was highly expressed and starch accumulated in meristematic tissues such as apical meristems and floral primordia, suggesting that local accumulation of starch could be necessary, in addition to the direct supply of Suc from source organs, to maintain the high growth rate characteristic of meristems.

GBSSI expression showed specific patterns during flower development. Expression in petals and sepals was visible at early developmental stages (Fig. 3, F and G) but was absent before anthesis. However, accumulation of starch granules in sepals and petals at this stage was detected by iodine staining (Fig. 4). Recently, plastid ontogeny during petal development in Arabidopsis was described (Pyke and Page, 1998). Young petals from unopened buds contain green chloroplasts, but as the petal lamina develops and expands plastids lose their chlorophyll and redifferentiate into leukoplasts. The loss of *GBSSI* expression observed in snapdragon petals before anthesis could also represent one aspect of the redifferentiation of chloroplasts into leukoplasts. Our results indicate that starch synthesized at earlier stages is accumulated in plastids and may subsequently be used by these organs as a C and energy source.

Expression of the *GBSSI* gene in carpel tissue was maintained throughout flower development and differentiation of the capsule. The actual accumulation of starch in this organ was confirmed by iodine staining, which showed large accumulation of starch granules in the funiculus, connecting the ovule to the ovary wall. Further studies of the rate of starch synthesis and mobilization will be necessary to determine the role of starch found in this organ in the process of fruit formation. This could give new insights to understand some of the alterations detected in starchless mutants of Arabidopsis, in which a low seed biomass accumulation was observed (Schulze et al., 1994).

Starch synthesis in leaves is tightly regulated through the so-called C-partitioning process, which diverts newly fixed CO_2 to Suc in the cytoplasm or starch in the chloroplast, depending on the levels of different metabolites that act as allosteric regulators (for review, see Stitt and Quick, 1989). Regarding the biosynthesis of leaf starch, it has been shown that this allosteric control is exerted on the first enzyme, ADP-Glc pyrophosphorylase. This enzyme is allosterically activated by 3-phosphoglycerate and inhibited by inorganic phosphate (for review, see Preiss and Levi, 1980; Preiss and Sivak, 1996). This allosteric control could account for the fluctuations of starch synthesis during the diurnal period. Li et al. (1992) showed circadian regulation of starch accumulation in sugar beet leaves. They observed that starch synthesis stopped during the night even when plants were kept under constant illumination, suggesting an endogenous circadian shift in C allocation at the end of the day, which diverts newly assimilated C to Suc synthesis and increases export at the expense of starch accumulation. Similar results were obtained for the C translocation during the day in the wild type and a starch-deficient mutant of tobacco (Geiger et al., 1995). However, the specific mechanism responsible for this response has not been identified.

Circadian regulation of other components of the C-partitioning process has also been shown as modulation of Suc phosphate synthase activity by a protein phosphatase (Jones and Ort, 1997). In this report we have shown for the first time, to our knowledge, circadian regulation of the mRNA abundance of a starch biosynthesis gene. Transcript levels of *GBSSI* decreased at the end of the day and were almost abolished during the night. This response was controlled by an endogenous circadian clock, which could be observed when the light was extended to the night period (Fig. 5B). This result suggests that the preparation of the plant for the usual night period is more complex than has

been previously suggested, involving changes in C allocation from chloroplasts to the cytosol, Suc synthesis, and expression of some of the starch biosynthesis genes. Expression of *GBSSI* during the day/night cycle was differentially regulated in distinct organs of the plant. Thus, although expression in leaves was controlled by an endogenous circadian clock, this mechanism did not operate in roots (Fig. 6), reflecting the differences in C metabolism between aerial, source organs such as leaves and dark, sink organs such as roots. Synthesis of starch involves different enzymes, and it will be interesting to establish whether there is coordinate regulation of the other starch biosynthesis genes.

Sequence analysis has revealed the presence of *cis* elements in the promoter region of snapdragon *GBSSI* (data not shown), which have been previously shown to be involved in circadian control of gene expression, such as the Circadian Clock Associated 1-binding site (Wang et al., 1997; Wang and Tobin, 1998), and light regulation, such as the binding site for the GT-1 transcription factor (Hiratsuka et al., 1994) or the G-box (Chattopadhyay et al., 1998). Similar elements are also present in the promoter region of the *SBE2.1* gene of Arabidopsis encoding a type II starch-branching enzyme and that has been recently shown to be light regulated (Khoshnoodi et al., 1998). The presence of these elements in the *GBSSI* promoter region suggests that fluctuations on the *GBSSI* mRNA abundance observed in leaves could be mediated by a circadian regulation of the gene transcription. However, a further functional analysis of the *GBSSI* promoter will be necessary to establish the role of those putative regulatory elements on the circadian regulation of *GBSSI* expression, as well as a possible light-mediated regulation of *GBSSI* expression.

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