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Sugar-Dependent Expression of the *CHS-A* Gene for Chalcone Synthase from Petunia in Transgenic *Arabidopsis*

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ABSTRACT

Transgenic *Arabidopsis thaliana* plants were constructed by introduction of a fusion of the gene for β-glucuronidase (GUS) to the *CHS-A* gene, which is one of the two genes for chalcone synthase that are actively expressed in the floral organs of petunia. The expression of the fusion gene *CHS-A::GUS* was low in transgenic *Arabidopsis* plantlets, but it was enhanced when plantlets or detached leaves were transferred to a medium that contained 0.3 molar sucrose, glucose, or fructose. No enhancement was observed when plantlets were transferred to a medium that contained 0.3 molar mannitol. Measurements of cellular levels of sugars revealed a tight linkage between the level of expression of the *CHS-A::GUS* gene and the level of accumulation of exogenously supplied sugars, in particular sucrose. The parallelism between the organ-specific accumulation of sugar and the organ-specific expression of the *CHS-A::GUS* gene was also observed in petunia and *A. thaliana* plants grown under normal conditions in soil. The consensus sequences for sugar responses, such as boxes II and III in members of the family of sporamin genes from the sweet potato, were found in the promoter region of the *CHS-A* gene that was used for fusion to the *GUS* gene. It is suggested that the expression of the *CHS-A* gene is regulated by sugars, as is the expression of other sugar-responsive genes, such as the genes for sporamin. A putative common mechanism for the control of expression of "sugar-related" genes, including the *CHS-A* gene, is discussed.

Chalcone synthase is a key enzyme in the synthesis of anthocyanin. Anthocyanin plays an important role in higher plants. The coloration of flower petals (or other related organs) by anthocyanin is most important for the attraction of pollinators, such as insects or birds. Many higher plants have developed specific patterns of coloration of their flower petals by anthocyanin. The gene for chalcone synthase has been studied for the most part as a stress-inducible gene in cell-culture systems. All known genes for chalcone synthase are light-inducible (being UV-inducible, in particular) or elicitor-inducible (3, 20, 23, 25, 31). *Cis* elements necessary for the induction by UV light have been identified in several species (20, 23).

It has long been suggested that sugars are involved in the synthesis of leaf or flower anthocyanin, as in the autumn reddening of tree leaves (9), but experimental results to support this hypothesis are few in number. Weiss and Halevy demonstrated the effect of sucrose, in the presence of gibberellin, on the appearance of anthocyanin pigment in stamen-detached flowers (28). Koes *et al.* (18) isolated the cDNA and a genomic clone that encode an isozyme of CHS[2] (CHS-A) as a petal-specific gene from petunia (16, 18). They also discovered that there are at least seven genes for isozymes of chalcone synthase in petunia, including *CHS-A* and *CHS-J*, which are expressed in petals (17, 18).

In this report, we describe an important aspect of the regulation of expression of the *CHS-A* gene, which was initially observed in transgenic *Arabidopsis* plantlets and may be operative in the original host, *Petunia hybrida*. The *CHS-A* gene from petunia seems to be regulated predominantly by the concentration of available sugars. Similar sugar-responsiveness is known from other systems such as the genes for sporamin (4), for β-amylase (5), for patatin (1, 13), and for proteinase inhibitor II (14, 15).

MATERIALS AND METHODS

Construction of a Chimeric *CHS-A::GUS* Gene

A 1-kb fragment of the CHS-A genomic clone VIP66 (kindly supplied by Dr. J.N.M. Mol of Free University, Amsterdam; ref. 18), extending from the *Eco*RI site at approximately position −820 to the *Bcl*I site at position +170, as measured from the site of initiation of transcription, was ligated into the *Eco*RI-*Bam*HI sites of plasmid pBluescript KS(+) (Stratagene, La Jolla, CA). The *Hin*dIII-*Xba*I fragment from the resultant plasmid, pHTS7, which included the subcloned 1-kb fragment, was ligated into the *Hin*dIII-*Xba*I site of plasmid pHTS6.2 (a derivative of plasmid pBI101.2 [11] with the gene for hygromycin phosphotransferase in its T-DNA region). The resulting plasmid pHTS10 was introduced into *Agrobacterium tumefaciens* strain C58C1 Rif^r(pGV2260) via triparental mating with *Escherichia coli* strain DH1 (pRK2013) as helper (2).

Plants

The following plants were used: *Arabidopsis thaliana* (L.) Heynh. ecotype Columbia and its mutant derivative GPR1

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[2] Abbreviations: CHS, chalcone synthase; kb, kilobase; MS, Murashige-Skoog; GUS, β-glucuronidase.

(*an, gl-1, th-1, tt-4*), and a *Petunia hybrida* F_1 line (Violet 23 × Red 51, *An4/an4, Bl/bl, Fl/fl, gpiB1/gpiB2, Hf1/hf1, Ph1/ph1, Po/po, Rt/rt*) (27).

Growth of Plants and Plant Cells

Seeds of *A. thaliana* were sterilized in a solution of NaClO ($Cl^- > 0.25\%$, plus Triton X-100 at a final concentration of 0.02% [v/v]) for 5 min and then washed twice with sterilized water. Basal culture medium (MS0, pH 6.5) was composed of Murashige-Skoog inorganic salts (22) supplemented with 2% (w/v) sucrose, 3 mg/L thiamine-HCl, 5 mg/L nicotinic acid, and 0.5 mg/L pyridoxine HCl. Gellangum (0.2% [w/v], San-ei Kagaku Kogyo Co., Ltd., Osaka, Japan) was added for preparation of plates. MS101 plates containing 1 mg/L benzyl adenine and 0.1 mg/L α-naphthyl acetic acid in MS0 were used for induction of callus and redifferentiation of infected explants. Rooting of regenerated shoots was achieved on MS-IAA plates that contained 0.5 mg/L of IAA in MS0.

The suspension culture of *Nicotiana alata* used as the nurse culture was initiated and maintained in MS-2,4-D liquid medium that consisted of 1 mg/L 2,4-D in MS0.

Regenerated and rooted plantlets were transferred to small plastic pots with rockwool and/or vermiculite moistened with MGRL medium, which contained, per liter: NaH_2PO_4, 1823 mg; Na_2HPO_4, 373 mg; $MgSO_4$, 1823 mg; KNO_3, 306 mg; Fe(III) EDTA, 5 mg; $Ca(NO_3)_2 \cdot 4H_2O$, 472 mg; $MnSO_4 \cdot 4H_2O$, 2.3 mg; H_3BO_3, 1.85 mg; $ZnSO_4$ $7H_2O$, 0.29 mg; $CuSO_4 \cdot 5H_2O$, 0.24 mg; $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.03 mg; $CoCl_2 \cdot 6H_2O$, 0.03 mg. Thiamine-HCl at 1 mg/L was added to the medium for growth of the thiamine-requiring mutant of *A. thaliana*.

Construction of Transgenic Plants

The leaf-disc method (8) was employed with the following modifications. Infected explants were grown on MS101 plates with a feeder layer. These plates were prepared by seeding MS101 plates with *N. alata* cells in suspension with sterilized filter papers on top of the cells. After 2 d of culture on such plates, explants were transferred onto new MS101 plates prepared with 0.5 mg/mL Claforan (Hoechst-Japan, Tokyo, Japan) for the elimination of *Agrobacterium* cells and then they were cultured for an additional 2 d. Thereafter, transgenic plants were selected on fresh MS101 plates that contained 0.5 mg/mL Claforan and 20 μg/mL hygromycin B (for *Arabidopsis*) or 50 μg/mL kanamycin (for petunia). Regenerated shoots were rooted on MS-IAA plates.

Four lines of kanamycin-resistant petunia plants were obtained with low numbers of copies of the 3.5-kb (*Eco*RI fragment of pHTS10) intact fusion gene. The *CHS-A::GUS* fusion gene exhibited flower-specific expression. It was apparent, therefore, that the promoter-proximal 5′ sequence of 1 kb included the necessary information for the petal-specific expression of the *CHS-A* gene. This result is consistent with the results of experiments by Koes *et al.* (19).

Forty-five hygromycin-resistant regenerated lines of *Arabidopsis* were obtained and the transgenic lines were named AtHT-1 through 45. Twenty-six of these lines gave enough seeds for further analysis and 24 strains had detectable GUS activity in flowers and leaves. Examination of the pollen revealed that 12 transgenic lines were probably 2n (diploid) plants in terms of ploidy. Southern blotting analysis of *Hin*dIII-digested, whole-plant DNA revealed that four strains, namely AtHT2, 9, 16, and 45, had low copy numbers (one to three copies per diploid) of introduced genes. Lines that were homozygous for the transgene were chosen from T_2 generations, they were maintained by self-pollination and were used for further studies.

Assay for GUS Activity

Extracts from samples of plant tissues were prepared and examined for GUS activity as described elsewhere (11). Protein was determined using a Protein Assay Kit (Bio-Rad Japan, Tokyo, Japan).

Histochemical Analysis of GUS Activity

The assay solution was a slightly modified version of that described by Jefferson *et al.* (11, 12). Ten milligrams of 5-bromo-4-chloro-3-indolyl-β-D-glucuronide were dissolved in 20 μL of DMSO, and 10 mL of 50 mM sodium phosphate buffer (pH 7.0) were added. Triton X-100 was then added at final concentration of 0.05% (w/v). Staining was performed as follows: Tissues obtained by manual sectioning were transferred to the incubation medium (vacuum infiltration was included in the case of thick sections) and sections were incubated at 37°C. After staining, Chl was eliminated by washing with 70% (v/v) ethanol.

Loading of Sugars and Other Substances

The samples were seedlings (14–20 d after imbibition) of transgenic *Arabidopsis* cultured on MS0 plates at 22°C with continuous illumination. Two methods were used to load sugars. In one method, whole plantlets were used; roots of the plantlets were dipped in a test medium and plantlets were cultured under these conditions. In the other method, detached rosette leaves from seedlings were used; the leaves were floated in test media and incubated for 3 d. Half-strength MS0 liquid medium was used as basal medium for loading experiments with sugars. Final concentrations of sugars ranged from 0 to 0.3 M. GA_3, when used, was added at final concentrations up to 1 mM. Inhibitors of the synthesis of gibberellin, namely paclobutrazol, uniconazol, ancymidol, and daminozide (B9), were used at final concentrations of 10 μM, 10 μM, 100 μM, and 1 mM, respectively. In these concentrations, seed germination of *A. thaliana* was completely inhibited and recovered by addition of GA_3.

Analysis of Sugars by HPLC

Sugars were extracted from plant tissues by grinding in hot (70°C) 70% (v/v) ethanol in 1.5-mL disposable plastic tubes with disposable pestles (Kontes Scientific Glassware Instruments, Vineland, NJ). After extraction for 10 min, samples were dried by vacuum desiccation. The resultant powder was dissolved in water and the solution was centrifuged (16,000 rpm, 10 min, 4°C). Supernatants were collected and used as

samples. These samples were analyzed on an HPLC system equipped with a pump, Hitachi L-6200; a refractive index monitor, Hitachi 655A-30; a column oven, Hitachi 655A-52, at 60°C; a column, GL-C610 combined with GL-C614; an autosampler, Hitachi 655A-40; and a computer for analysis, Hitachi D-2000. The carrier solvent was water and the flow rate was 1 mL/min.

RESULTS

Examination of Transgenic *Arabidopsis*

Four lines of transgenic *Arabidopsis* (AtHT 2, 9, 16, and 45) grown in normal soil were examined to determine whether organ specificity was conserved as in the case of transgenic petunias (19). The flowers of *A. thaliana* are very small and it was practically impossible to separate the floral organs. Therefore, we measured the activity of GUS in extracts of both flowers and floral buds that included sepals. As shown in Figure 1, little enzymatic activity was detected and the flower-specific expression of the *CHS-A::GUS* fusion gene was not significant in the extracts of flowers and buds of transgenic *Arabidopsis* plantlets. Histochemical staining was carried out in order to examine the organ specificity and the various patterns of low-level expression (Fig. 2). We were able to detect significant and reproducible staining in the leaf, the sepal, the stigma, the filament, and the young seed coat. The stem was slightly stained. By contrast, flower petals were not stained under these conditions. No organs or tissues of wild-type plants and transgenic plants that carried a promoterless *GUS* gene were stained at all (data not shown).

Induction of the Expression of the *CHS-A::GUS* Fusion Gene by Various Sugars

During the course of this study, we found that the activity of GUS in rosette leaves of transgenic *Arabidopsis* plantlets grown *in vitro* in MS0 (which contained 3% sucrose, *i.e.* 0.08 M sucrose) was higher than that in leaves of the plants grown in soil. In order to examine the effects of an exogenous supply of various sugars, leaves were detached from young seedlings (2 weeks old) and were transferred to half-strength MS0 liquid medium that contained one of a variety of sugars at 0.3 M. The GUS activity of rosette leaves increased by as much as 10-fold in the presence of 0.3 M sucrose after 3 d (Fig. 3). In this system, using detached leaves, the presence of 0.3 M glucose, 0.3 M fructose, or 0.3 M maltose also caused an increase of 5- to 10-fold in GUS activity, but the effects were lower than that of sucrose. The effect of 0.3 M galactose was very small (Fig. 3). The preparations of sugars contained low levels (below 0.5%) of contaminating sucrose or glucose. However, such a correspondingly low concentration of authentic sucrose (2 mM) did not stimulate the expression of the *CHS-A::GUS* fusion gene (data not shown). Therefore, the effects obtained as a result of the treatment with such sugars were thought to be the effects of the sugars themselves. When transgenic *Arabidopsis* plantlets were treated with 15 mM sucrose, the treated leaves showed twofold higher GUS activity than that of leaves that were not treated with sugar (for all four transgenic lines; data not shown). Thus, the effect obtained with 15 mM sucrose corresponded with that of 0.3 M of galactose. By contrast, no increase in GUS activity was detected with 0.3 M mannitol or without addition of sugars after 3 d or longer in culture.

The sugars did not affect the roots and the cells of roots did not express GUS activity, even after the inclusion of these sugars, when whole plantlets were treated.

The leaves from transgenic *Arabidopsis* carrying a fusion of the promoter for 35S RNA from cauliflower mosaic virus and the *GUS* gene were incubated in basal medium or in the presence of 0.3 M mannitol or sucrose. 4-Methylumbelliferone was released (3.4 ± 0.2 nmol min^{-1} mg^{-1} extracted protein; $n = 3$) from leaves incubated in basal medium and 3.3 ± 0.6 nmol min^{-1} mg^{-1} extracted protein ($n = 3$) was released from mannitol-treated leaves. In leaves treated with 0.3 M sucrose, this value was 1.9 ± 0.6 ($n = 3$). The promoter for 35S RNA from cauliflower mosaic virus, therefore, was not affected by the addition of exogenous sugars.

The presence of either glucose, sucrose, or fructose at 0.3 M resulted in the production of anthocyanin, as revealed by the development of purple coloration of leaves during the incubation period. During the culture, the pH of the culture medium did not show any major variation under the conditions tested and ranged from 5.1 to 5.3.

To extend the above findings obtained with detached leaves, we next examined the effects of exogenously supplied sugars on intact plantlets. As can be seen in Figure 4, glucose and sucrose stimulated the activity of GUS in leaves in this intact plant system.

Intracellular concentrations of sugars in leaves were then analyzed by HPLC after treatment of transgenic *Arabidopsis* with each sugar. We were able to detect fructose, glucose, maltose, and sucrose as major components of the free sugars taken up by *Arabidopsis* grown *in vitro*. As can be seen in Figure 4, the increase in the concentration of sucrose apparently coincided with the increase in GUS activity in *Arabidopsis* (line AtHT16). The concentration of glucose was al-

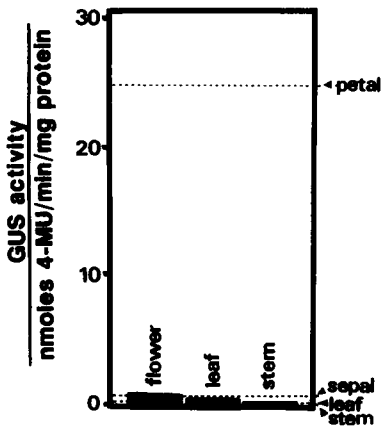

Figure 1. Patterns of expression of the *CHS-A::GUS* fusion gene in transgenic *Arabidopsis*. The GUS activity of transgenic *Arabidopsis* plant is shown for each organ. That of organs of transgenic petunia plants is shown by arrowheads on the right side of the panel for reference. Each value is the mean of three replicates.

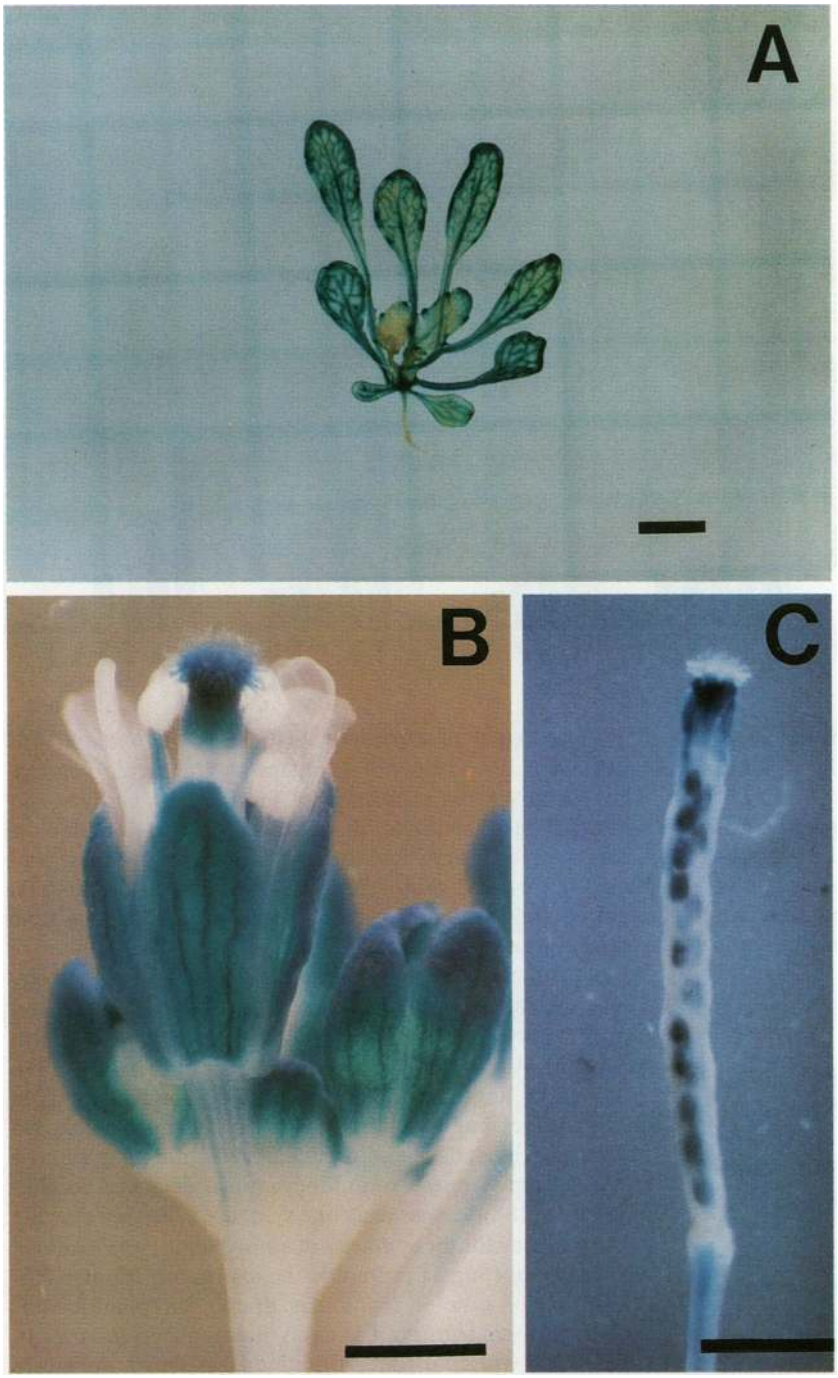

Figure 2. Histochemical staining of transgenic *Arabidopsis*. Plantlet (A), flower (B), and young fruit (C) stained with a solution of 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (see text) that contained 0.05% Triton X-100 at 37°C. Chl was removed by treatment with 70% ethanol. Bar = 5 mm for A, and 1 mm for B and C.

ready high after 1 d of growth with 0.3 M glucose or 0.3 M sucrose. The amounts of fructose and maltose in treated plants were apparently not correlated in any way with the GUS activity.

Organ-Specific Accumulation of Sugars

At the onset of the above observations, we hypothesized that the organ-specific expression of the *CHS-A* gene was associated with an organ-specific accumulation of sugars in the original host, petunia. Therefore, we examined the distribution of the major sugars in each organ obtained from intact petunia plantlets, cultivated in pots with soil but without the application of sugars. As shown in Figure 5, petunia clearly showed petal-specific accumulation of glucose and fructose. The levels of these sugars were dependent on the ages of flower buds, and the difference between minimum and maximum values was about 20-fold. The level of sucrose in petals was higher than that in other organs, but it did not show an age-dependent increase, as it did for glucose and fructose. The level of maltose was very low (not shown). The time at which the level of sugars (glucose and fructose) reached a peak was

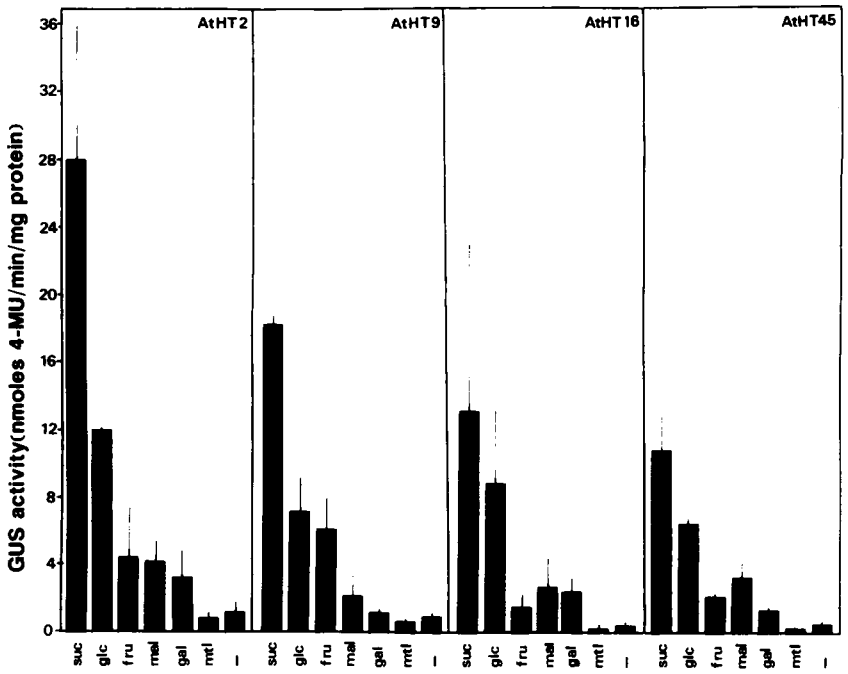

Figure 3. GUS activity in transgenic *Arabidopsis* carrying the *CHS-A::GUS* fusion gene after the addition of various sugars. Leaves detached from seedlings of transgenic *Arabidopsis* AtHT 2, 9, 16, and 45 were transferred to a sugar-containing medium and GUS activity in (rosette) leaves was measured after 3 d. The basal medium used for the control contained no sugar. From left to right, sucrose (suc), glucose (glc), fructose (fru), maltose (mal), galactose (gal), mannitol (mtl) and no sugar (—) treatment. All sugars were used as 0.3 M solutions. Data are the means of three replicate experiments. Vertical bars indicate half of each standard error.

nearly same as the time at which the expression of the *CHS-A::GUS* fusion gene was maximum. In contrast, the sepals of petunia showed no change in sugar content.

In the case of *A. thaliana* plants grown in pots, sucrose was the only sugar detected at a significant level and no organ-specific accumulation of sugars was detected, irrespective of the growth stage (see Fig. 5D). Fructose and maltose were not detected in any organs and glucose was detected only in stems at low levels (0.49 μmol/g fresh weight).

Effect of Gibberellin

Because Weiss *et al.* (29) clearly demonstrated the effects of GA_3 on the expression of anthocyanin-related genes, we examined the effect of GA_3 in our system. We used both detached leaves floated in liquid half-strength MS0 medium with GA_3 and detached inflorescences floated in the same media. In both experiments, we could not detect any significant enhancement of the expression of the *CHS-A::GUS* gene by the addition of GA_3. We also examined various combinations of sucrose, GA_3, and inhibitors of the synthesis of gibberellin as described in "Materials and Methods." The addition of GA_3 and/or paclobutrazol did not appear to make a significant difference on the effect of the addition of sucrose alone in our experimental system.

DISCUSSION

The *CHS-A* gene was initially isolated as a petal-specific gene from *Petunia hybrida* (18). The 1-kb fragment of the *CHS-A* gene, which is expressed in petals specifically (16, 18), was introduced into *Arabidopsis thaliana* in the form of a *CHS-A::GUS* fusion in order to examine whether the organ specificity is conserved in other plant species. We chose this host because *A. thaliana* is an excellent system for plant molecular biology for the many reasons described elsewhere (21). The extent of expression of GUS in *A. thaliana* was lower than in corresponding organs of transgenic petunia. Thus, petal-specific expression of the *CHS-A* gene was not conserved in *A. thaliana*. Because *A. thaliana* does not develop colored flowers but carries one gene for chalcone synthase (3), this observation suggests the absence of the factor(s) required for the petal-specific expression of the chalcone synthase gene from petunia.

The synthesis of anthocyanin is regulated by many factors. Stress, such as high-intensity light, in particular UV light, and nitrogen starvation, is known to induce the synthesis of anthocyanin. Additionally, sugars are believed to regulate the synthesis of anthocyanin in the case of the reddening of autumn leaves, but no supporting evidence for such regulation has been provided. Weiss and Halevy found that sucrose enhanced the synthesis of anthocyanin in detached corollas of petunia (28). Because chalcone synthase is the key enzyme in the pathway for the synthesis of anthocyanin, we examined whether or not sugars promoted the expression of the *CHS-A::GUS* fusion gene. *A. thaliana* is an excellent plant for this kind of analysis because it is easily manipulated *in vitro*. In experiments with transgenic *Arabidopsis*, as shown in Figures 3 and 4, exogenously applied sugars were taken up by plants and the time courses of the increase in GUS activity and the increase in sucrose content were very similar. Because the level of glucose and of fructose rapidly reached a high plateau value, glucose and fructose also appear to be intimately involved in the expression of the *CHS-A* gene. The increased cellular synthesis of anthocyanin in leaves was also observed in *A. thaliana* under these conditions, and the *CHS-A::GUS* fusion gene was simultaneously expressed at high levels. Thus, we have extended the previous findings by Weiss and Halevy (28).

Weiss *et al.* (29) showed clearly that GA_3 plays a major role in the synthesis of anthocyanin-related proteins, such as chal-

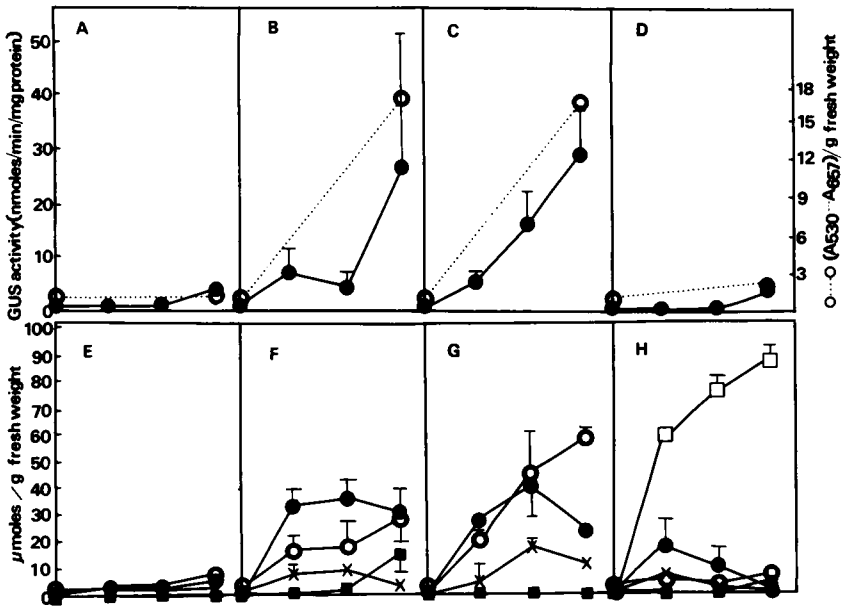

Figure 4. Effects of the addition of glucose, sucrose, and mannitol on GUS activity and sugar contents. Seedlings of transgenic *Arabidopsis* AtHT 16 were grown in MS0 medium (3% sucrose) and were then transferred to half-strength MS0 supplemented with various sugars. Levels of sugars in leaves were measured by HPLC during the first 3 d and anthocyanin was extracted from leaves with HCl-methanol and quantitated as (A_{530} minus A_{657})/g fresh weight. Panels A through D show the GUS activities (●) and amounts of anthocyanin (○) measured after treatments with various sugars. Panels E through H show levels of sugars. Sugars added to the basal medium were: A, E, none; B, F, 0.3 M glucose; C, G, 0.3 M sucrose; and D, H, 0.3 M mannitol. In panels E through H, the following symbols are used for each sugar: ○, sucrose; ●, glucose; ×, fructose; □, mannitol; and ■, maltose. Vertical bars, absent in the case of the amount of anthocyanin, indicate half of each standard error (n = 3).

cone synthase and chalcone flavanone isomerase of *P. hybrida,* in a stamen-detached corolla system. Therefore, we examined the effect of GA_3 using two different materials from transgenic *Arabidopsis.* The effect by GA_3 did not appear to be significant in the expression of the *CHS-A::GUS* fusion gene in leaves and flower buds. In our experimental systems, GA_3 does not play an important role. The report that seedlings of radish also seem to have a mechanism for GA-independent synthesis of anthocyanin (10) is consistent with our observations.

Considering the parallels between the activity of the CHS-A promoter and the accumulation of sugars in transgenic *Arabidopsis,* we examined the possibility that the organ-specific expression of the gene can be attributed to the organ-specific distribution of sugars in petunia and *A. thaliana.* The organ in which the CHS-A promoter was active at high levels was found to accumulate sugars at high concentrations. The activity of GUS in flowers reached a peak just before anthesis, at the same time as glucose and fructose were observed to be present at their highest concentrations in flowers. Levels of sucrose in flowers were also higher than those in leaves or sepals. Sucrose is known as the translocating sugar in many plant species (7). It is possible, therefore, that sucrose is translocated to the petals and is then cleaved to monosaccharide components, glucose and fructose, both (or one) of which are (or is) responsible for the expression of the *CHS-A* gene. A kinetic study might reveal the respective roles of sucrose, fructose, and glucose. In contrast with petunia, *A. thaliana* showed no clear organ-specific accumulation of endogenous sugars. The low concentration of sugars appeared to be correlated with the less specific and low level of expression of the *CHS-A::GUS* fusion gene in this plant species. The root was an exception in that the *CHS-A::GUS* fusion gene was not expressed even after the treatment with sugars. From our results, it appears that the synthesis of anthocyanin may be promoted by high intracellular levels of sugars in the plants

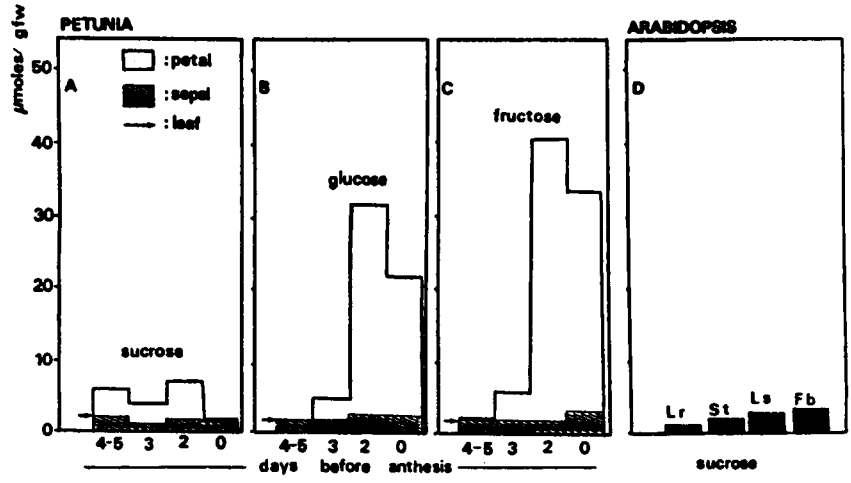

Figure 5. Organ-specific accumulation of sugars during flowering. Panels A, B, and C show concentrations of the following sugars in petunia: A, sucrose; B, glucose; C, fructose. Open bars show sugars in floral petals and hatched bars show sugars in sepals. Arrows on the left side of panels indicate the level of each sugar in the leaf. The results are shown for plants as they approached the time at which flowers open. Panel D shows the concentration of sucrose in each organ of *A. thaliana* (30 d after sowing). Lr, leaf of rosette; St, stem; Ls, leaf on stem; Fb, flowers including sepals and flower buds. Each result is the average of results from three experiments.

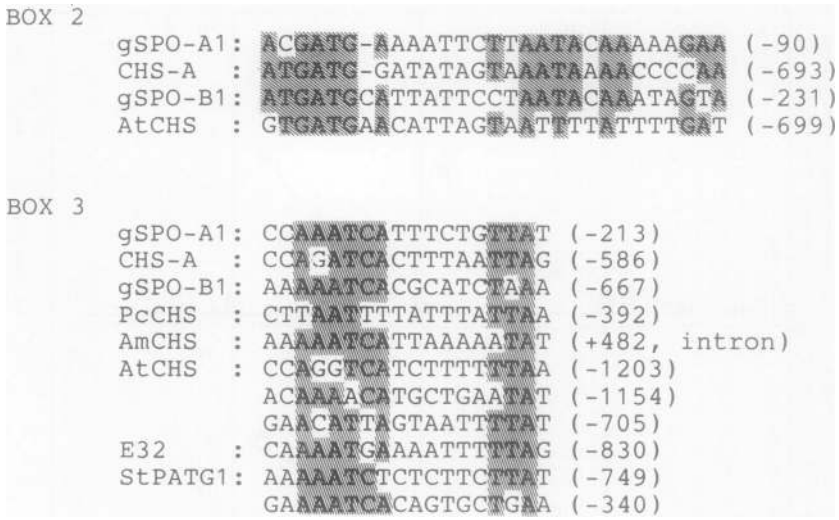

Figure 6. Homologous sequences associated with the "sucrose boxes" in the 5′ flanking regions of gSPO-A1 and the B1 clones of sporamin gene from sweet potato (5). The boxes are aligned to the sequences of CHS-A. AtCHS, the gene for chalcone synthase from *A. thaliana* (3); PcCHS, the gene for chalcone synthase from parsley (6); AmCHS, the gene for chalcone synthase from snap dragon (24); E32, the gene for wound-inducible proteinase inhibitor II from potato (15); and StPATG1, the gene for patatin from potato (1). Identical nucleotides are indicated by shadowing. The numbers in parentheses are the nucleotide numbers from each site of initiation of transcription, which is taken as +1.

examined. Reddening of leaves in autumn may be an example of such regulation. The identification of a sugar-responsive *cis* element of the *CHS-A* gene will provide final proof of this hypothesis.

The members of the sporamin gene family (which belongs to the tuberous-root-storage-protein gene) of the sweet potato are known as organ-specific (tuber-specific) genes and are expressed at high levels when high concentrations of sucrose are applied to stems (4). The β-amylase gene from the sweet potato is also a tuber-specific and sugar-responsive gene. The consensus sequences for responses to sugars, such as boxes II and III (5), are present in the upstream region of these genes. Using a computer system in a search for homologies, we found sequences similar to each box in the promoter of the *CHS-A* gene used in the present study (Fig. 6). The *chs* genes from *Petroselinum crispum, Antirrhinum majus* and *Arabidopsis thaliana* also belong to this family (see also, Fig. 6). *A. thaliana* did, in fact, show sugar-dependent accumulation of anthocyanin in this study. We have also identified such box-like sequences in other sugar-responsive genes, such as the gene for patatin in potato (1, 13) and the gene for proteinase inhibitor II in potato (14, 15), also shown in Figure 6. The gene for proteinase inhibitor II is not expressed in the root (14), as shown similarly for the *CHS-A* gene in the present study. The existence of similar "sugar boxes" leads us to suspect a common molecular mechanism of sugar-related regulation. van der Meer *et al.* (25) analyzed the 5′-sequence of the *CHS-A* gene using transgenic petunia. The minimum requirement for the floral specificity was localized to a 67-bp region upstream from the RNA cap site. The region from −800 to −530, which contained the box II sequence, seemed to have an enhancing effect. Deletion analysis should be performed from the standpoint of control by sugars in transgenic *Arabidopsis* plants.

The implications of the apparent sugar-dependent control have been examined, in the case of genes for sporamin and patatin, with reference to sink-source theory (4, 13, 30). However, in such studies the concentrations of the translocated sugars were not appropriately analyzed. We measured the concentrations of free sugars that were taken up and showed a close correlation between the CHS-A activity and the level of sugars. This phenomenon can be understood as follows. Because the petunia is pollinated by insects, its reproductive strategy involves the development of large petals and systems for the translocation of sugars to organs that do not assimilate sugars, such as petals and nectar glands. These flowers should attract pollinators. *A. thaliana* is not pollinated by insects and is self-fertilized, so it does not need a large petal nor does it need a petal-specific sugar-translocation system and, in fact, it has a small, white petal.

Light-dependent expression of the *CHS-A* gene was also demonstrated in our transgenic plants (data not shown). The expression of the petunia CHS-A promoter is regulated by at least three mechanisms: a light-dependent mechanism; a non-root, organ-specific mechanism; and a sugar-related mechanism. Further analyses, such as the deletion of specific 5′ fragments of DNA, will reveal further details of these regulatory networks.

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