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Sugar-Inducible Expression of a Gene for β-Amylase in *Arabidopsis thaliana*[1]

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The levels of β-amylase activity and of the mRNA for β-amylase in rosette leaves of *Arabidopsis thaliana* (L.) Heynh. increased significantly, with the concomitant accumulation of starch, when whole plants or excised mature leaves were supplied with sucrose. A supply of glucose or fructose, but not of mannitol or sorbitol, to plants also induced the expression of the gene for β-amylase, and the induction occurred not only in rosette leaves but also in roots, stems, and bracts. These results suggest that the gene for β-amylase of *Arabidopsis* is subject to regulation by a carbohydrate metabolic signal, and expression of the gene in various tissues may be regulated by the carbon partitioning and sink-source interactions in the whole plant. The sugar-inducible expression of the gene in *Arabidopsis* was severely repressed in the absence of light. The sugar-inducible expression in the light was not inhibited by 3(3,4-dichlorophenyl)-1,1-dimethylurea or by chloramphenicol, but it was inhibited by cycloheximide. These results suggest that a light-induced signal and de novo synthesis of proteins in the cytoplasm are involved in the regulation. A fusion gene composed of the 5′ upstream region of the gene for β-amylase from *Arabidopsis* and the coding sequence of β-glucuronidase showed the sugar-inducible expression in a light-dependent manner in rosette leaves of transgenic *Arabidopsis*.

β-Amylase (EC 3.2.1.2) is abundant in some starch-storing organs of plants, such as seeds and tuberous roots. In addition to these reproductive organs, β-amylase is also present in various vegetative tissues (Beck and Ziegler, 1989). However, the physiological roles of β-amylase in plants in vivo are not known at present in spite of extensive enzymological studies of the breakdown of starch in vitro. Native starch granules are not attacked by plant β-amylases without the prior digestion by other enzymes or the solubilization by boiling (Beck and Ziegler, 1989). Precursors to β-amylases from plants (Kreis et al., 1987; Monroe et al., 1991; Yoshida and Nakamura, 1991) do not contain the N-terminal transit peptide sequences that are responsible for targeting proteins to plastids. Indeed, results of most of the previous studies of vegetative tissues from various plant species indicate that β-amylase is present outside the chloroplasts where the synthesis and accumulation of starch occurs (Beck and Ziegler, 1989). β-Amylases in the leaves of pea (Ziegler and Beck, 1986) and possibly in *Arabidopsis* as well (Monroe et al., 1991) may be present in the vacuoles.

β-Amylases of several plant species appear to be subject to highly complex regulation, and a better picture of the mode of expression of the gene for β-amylase should provide some clues to the physiological role of this enzyme. In mustard cotyledons, light induces the accumulation of mRNA for β-amylase, and this regulation is mediated by phytochrome (Sharma and Schopfer, 1987). Expression of a gene for β-amylase in sweet potato (Yoshida et al., 1992) is developmentally regulated in tuberous roots (Nakamura et al., 1991; Yoshida and Nakamura, 1991), and it is inducible in atypical tissues in response to high levels of Suc or other metabolizable sugars (Nakamura et al., 1991; Takeda et al., 1994). In addition, transcription of the gene for β-amylase of sweet potato is also responsive to oligosaccharides, such as polygalacturonic acid and chitosan, and to ABA (Ohto et al., 1992).

Rosette leaves of *Arabidopsis* contain at least three major amylases, and the extrachloroplastic β-amylase is the most abundant among them, accounting for about 80% of the total amylolytic activity (Lin et al., 1988). β-Amylase was found to accumulate in the leaves of several different mutants of *Arabidopsis* that were defective in the synthesis of starch or in the breakdown of starch when plants were grown with a 12-h light/dark cycle (Caspar et al., 1989; Monroe and Preiss, 1990). It was suggested that β-amylase may be induced in response to the high levels of soluble sugars that accumulate during the photoperiod in these mutants (Caspar et al., 1989).

The results presented in this report indicate that the expression of a single-copy gene for β-amylase in *Arabidopsis* can be induced in various vegetative tissues by an exogenous supply of high levels of Suc or other metabolizable sugars, a condition that also induces the accumulation of starch. Although the sugar-inducible expression of the gene for β-amylase was greatly enhanced by illumination, photosynthesis appears not to be required for such regulation. Analysis of the expression of a fusion gene, which consisted of the 5′ upstream sequence of the gene for β-amylase and the coding sequence of the gene for GUS in transgenic *Arabidopsis* suggested that the sugar-inducible

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Abbreviation: CaMV, cauliflower mosaic virus.

expression of the gene for β-amylase in the light is regulated at the level of transcription.

MATERIALS AND METHODS

Plant Materials

The Columbia ecotype of *Arabidopsis thaliana* (L.) Heynh. was used in most experiments. Transgenic plants were prepared from the WS ecotype of *A. thaliana*. *Arabidopsis* plants were grown under hydroponic conditions in a solution of sterile Murashige-Skoog salts that contained 1% Suc at 22°C under continuous fluorescent light at an intensity of 70 μmol m^{-2} s^{-1}. For treatment with Suc or other sugars, roots of 3-week-old plants were dipped in sterile solutions of sugars dissolved in water and they were then incubated under these conditions either under continuous light or in darkness at 22°C. Alternatively, mature leaves that had been cut from 3-week-old plants with a sharp razor blade were immersed in a solution of sugar at their cut edges. DCMU from Nacalai Tesque, Inc. (Kyoto, Japan), was dissolved in ethanol and diluted with water to a final concentration of 20 μM. Cycloheximide and chloramphenicol were obtained from Sigma.

Extraction of Proteins and Analysis of Enzymatic Activity

Extracts were prepared by grinding tissues in the cold extraction buffer composed of 50 mM Tris-HCl (pH 8.0), 5 mM EDTA, 0.25 M Suc, and 10 mM DTT. The homogenate was centrifuged at 15,000*g* for 10 min and the supernatant was used as the protein extract. The protein content of each extract was determined with a protein assay kit (Bio-Rad). Activity staining of β-amylase in polyacrylamide gels was carried out as described previously (Nakamura et al., 1991) except that after electrophoresis the gel was soaked in a 0.2% solution of soluble starch in 0.1 M sodium acetate (pH 4.8) at 37°C for 2 h. Total amylase activity, measured as the increase in concentration of reducing sugars, was assayed with soluble starch as the substrate (Morita et al., 1975). GUS activity was determined by the fluorimetric method described by Jefferson et al. (1987).

Cloning and Sequencing of DNA

A pair of degenerate oligonucleotides was designed on the basis of previously reported amino acid sequences of plant β-amylases for use in amplifying part of the protein-coding region of the gene for β-amylase from *Arabidopsis* by PCR. A set of 128-fold degenerate oligonucleotides (primer 5′) had the sequence 5′-AGAGATCTGTNATGGTNGAYGTNTGGTGGGG-3′, where N is G + A + T + C and Y is T + C. The underlined sequence indicates a *Bgl*II restriction site, and the 3′ proximal 23 nucleotides correspond to the peptide sequence Val-Met-Val-Asp-Val-Trp-Trp-Gly. A set of 192-fold degenerate oligonucleotides (primer 3′) had the sequence 5′-GGAATTCSRTYGGTGTAGAAMATRTCRWG-3′, where S is G + C, R is G + A, Y is T + C, M is T + A + G, and W is G + T. The underlined sequence indicates an *Eco*RI site, and the reverse sequence complementary to the 3′ proximal 23 nucleotides corresponds to the peptide sequence (His or Pro)-Asp-Ile-Lys-Tyr-Trp-(Asn or Asp)-(Arg or Gly). The above-described two peptide sequences are located within the same exon in the gene for β-amylase of sweet potato (Yoshida et al., 1992). An expected 240-bp-long fragment was amplified by PCR reactions with genomic DNA from *Arabidopsis* as the template. The nucleotide sequence of this fragment revealed an open reading frame that encoded a polypeptide with 74% sequence identity at the amino acid level to the β-amylase from sweet potato (Yoshida and Nakamura, 1991), and the nucleotide sequence was identical with that of the corresponding region of a cDNA for β-amylase of *Arabidopsis* reported by Monroe et al. (1991) while this work was in progress.

A λ-phage genomic library of *A. thaliana* Landsberg *erecta*, a kind gift from Dr. Kiyotaka Okada (National Institute for Basic Biology, Okazaki, Japan), was screened with the ^{32}P-labeled fragment from PCR as probe. Restriction endonuclease fragments of the genomic inserts from positive clones were subcloned into appropriate sites of pUC119 and Bluescript KS(+) vectors (Stratagene). An overlapping set of deletion subclones was generated by digestion with *Exo*III and *Exo*VII, and nucleotide sequences were determined for both strands with a dideoxynucleotide-sequencing kit from Takara Shuzo (Kyoto, Japan). Primer extension analysis was carried out using a 23-mer oligodeoxyribonucleotide, 5′-GCTTCTCGTTGTAATTGGTAGCC-3′, complementary to the sequence between positions +112 and +134 of the gene for β-amylase and poly(A)$^+$ RNA as described previously (Yoshida et al., 1992).

Extraction of DNA and RNA for Hybridization

Total genomic DNA from *Arabidopsis* was digested with restriction endonucleases, subjected to electrophoresis in a 0.7% agarose gel, and then transferred to a nylon membrane (Zeta probe, Bio-Rad). Hybridization was carried out with the ^{32}P-labeled fragment from PCR as a probe in 5× SSC, 5× Denhardt's solution, 0.1% SDS, 250 μg/mL salmon sperm DNA, 50 mM sodium phosphate (pH 7.5), and 50% deionized formamide at 43°C for 48 h. The membrane was washed in 0.1× SSC, 0.1% SDS at 43°C and subjected to autoradiography under an intensifying screen at −80°C.

Total RNA was prepared from rosette leaves of *Arabidopsis* and analyzed to determine the level of mRNA for β-amylase by northern blot hybridization, as described previously (Nakamura et al., 1991). Hybridization with the ^{32}P-labeled fragment from PCR was allowed to proceed for 17 h. Control hybridization with ^{32}P-labeled cDNA for α-tubulin, kindly provided by Dr. Peter Snustad of the University of Minnesota, was also carried out.

Construction of Plasmids and Transformation of Plants

The 5′ upstream region of the gene for β-amylase of *Arabidopsis* was isolated as a 1.7-kb *Hin*dIII fragment that also contained a small portion of the coding sequence (−1587 to +136 relative to the site of initiation of transcription). This *Hin*dIII fragment was cloned into the *Hin*dIII site

of pBI101 (Jefferson et al., 1987) in phase with the coding sequence of GUS to yield pABH. pABH was cleaved with *Eco*RI at a site downstream from the *nos* terminator, and a 4.8-kb *Hin*dIII fragment that contained the coding sequence for hygromycin phosphotransferase under control of the CaMV 35S promoter, a generous gift from Dr. Ko Shimamoto of Plantech Institute (Yokohama, Japan), was inserted by blunt-end ligation after a "filling-in" reaction. The resulting plasmid, pABH-Hm1, was transferred from *Escherichia coli* cells to *Agrobacterium tumefaciens* EHA101 (Hood et al., 1986) cells by triparental mating. The *Agrobacterium*-mediated transformation of *A. thaliana* ecotype WS was carried out as described elsewhere (Valvekens et al., 1988; Akama et al., 1992). *A. thaliana* transformed with pIG121-Hm that contained the CaMV 35S promoter:intron-GUS fusion gene (Ohta et al., 1990) has been described (Akama et al., 1992).

RESULTS

Sugar-Inducible Expression of a Gene for β-Amylase in *Arabidopsis*

Three-week-old *Arabidopsis* plants were cultured with a solution of Suc at various concentrations under continuous light for 3 d. Proteins were extracted from rosette leaves of more than seven plants, and total amylolytic activity was assayed. The amylolytic activity in rosette leaves was increased significantly by treatment of plants with 3 or 6% Suc (Fig. 1A). Amylolytic activities in rosette leaves of plants treated with 6% Suc were 2 to 4 times higher than those in plants treated with 3% Suc, depending on the particular experiment. Treatment of plants with 10% Suc also increased the amylolytic activity in rosette leaves, although the activity was lower than that in plants treated with 6% Suc (data not shown). Amylolytic activities in rosette leaves of plants treated with water were similar to those in nontreated plants. Proteins in the extracts were separated on nondenaturing polyacrylamide gels that contained soluble starch and stained for amylolytic activity in situ to detect the band of β-amylase protein (activity staining). The extracts from rosette leaves of nontreated plants and of plants treated with water for 3 d yielded one band of amylolytic activity when 7 μg of protein were applied to the gel (Fig. 1A). Judging from its activity and relative electrophoretic mobility, this band of amylolytic activity seems to correspond to the A3 isozyme of amylase in *Arabidopsis* leaves that had been characterized previously as an extrachloroplastic β-amylase (Lin et al., 1988). The band of β-amylase became significantly more intense after treatment of plants with 3 or 6% Suc (Fig. 1A). Similar increases in the level of amylolytic activity in the extract and in the activity of the β-amylase band were obtained when mature rosette leaves were excised from 3-week-old plants and supplied with a solution of Suc at their cut edges and incubated under continuous light (data not shown).

Total RNA was prepared from rosette leaves and the level of mRNA for β-amylase was analyzed by northern blot hybridization with the ^{32}P-labeled fragment, amplified

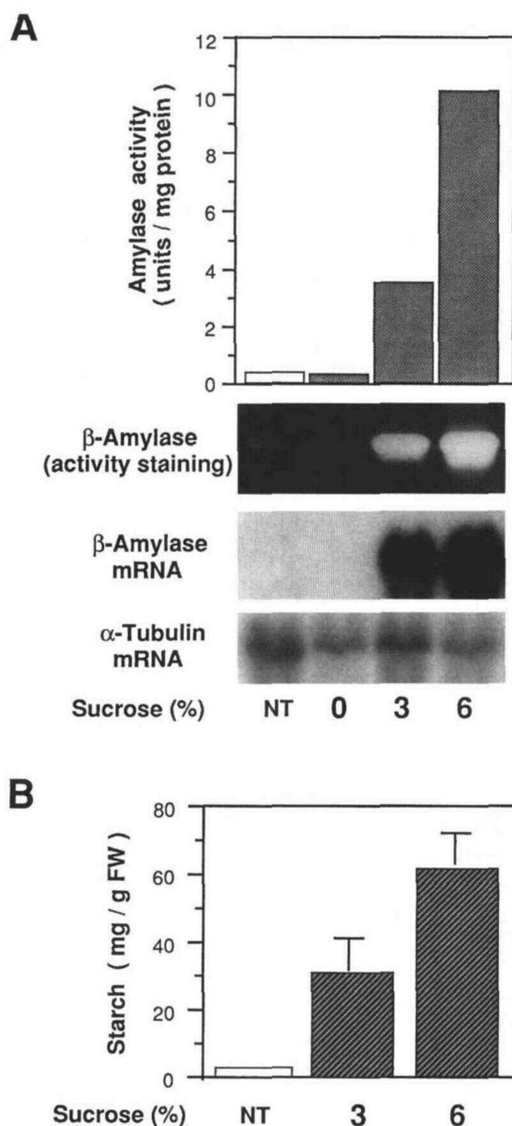

Figure 1. Suc-induced increases in levels of β-amylase (A) and starch (B) in rosette leaves of *Arabidopsis*. Roots of 3-week-old *Arabidopsis* plants were dipped in a solution of 0, 3, or 6% (w/v) Suc in water and incubated in this way for 3 d under continuous light. A, Proteins were extracted from rosette leaves of more than seven plants for each treatment and the level of β-amylase was assayed by measuring the total amylolytic activity and by activity staining for β-amylase in a polyacrylamide gel that contained soluble starch. Total amylase activity was expressed in terms of activity, where one unit corresponds to the amount of enzyme that releases 1 μmol of reducing sugar per min under the conditions of the assay. For activity staining of β-amylase, 7 μg of proteins were fractionated by gel electrophoresis. Total RNA was also isolated from rosette leaves and analyzed for the levels of β-amylase mRNA and α-tubulin mRNA by northern blot hybridization. Twenty micrograms of total RNA were loaded in each lane. B, *Arabidopsis* plants were treated with 3 or 6% Suc as described in A. Average results of three independent experiments are shown with SD. NT, Rosette leaves from nontreated plants; FW, fresh weight.

by PCR, of part of the coding sequence of the gene for β-amylase from *Arabidopsis* (see "Materials and Methods") as probe. As shown in Figure 1A, mRNA for β-amylase was hardly detectable in the mature rosette leaves of 3-week-old plants. A significant increase in the level of this mRNA occurred in plants that had been treated with 3 or 6% Suc for 3 d. Resembling the levels of amylolytic activity and the activity of the β-amylase band, the highest level of mRNA for β-amylase was obtained with 6% Suc (Fig. 1A), and treatment of plants with 10% Suc was less effective than that with 6% Suc (data not shown). Neither the electrophoretic patterns of RNAs after staining with ethidium bromide nor the level of mRNA for α-tubulin (Fig. 1A) differed significantly among the various preparations of RNA examined.

The Suc-induced accumulation of mRNA for β-amylase in sweet potato occurs concomitantly with the accumulation of a large amount of starch (Nakamura et al., 1991). The accumulation of starch also occurred in leaves of *Arabidopsis* when plants were treated with 3 or 6% Suc for 3 d under continuous light (Fig. 1B). Treatment of excised rosette leaves with Suc also resulted in the accumulation of starch (data not shown).

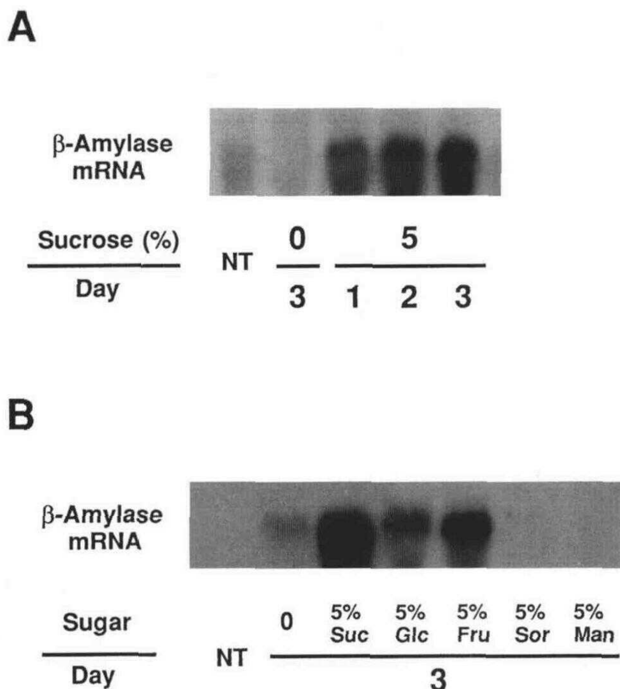

Figure 2. Time course of the induction of β-amylase mRNA in rosette leaves of *Arabidopsis* after treatment with Suc (A) and the induction of β-amylase mRNA by treatment with various sugars (B). Roots of 3-week-old plants were dipped in a solution of 5% Suc in water and incubated for 1, 2, or 3 d under continuous light (A), or they were dipped in a 5% solution of one of various sugars and incubated under continuous light for 3 d (B). Concentrations of the sugars used were as follows: 5% Suc, 146 mM; 5% Glc, 276 mM; 5% Fru, 276 mM; 5% sorbitol (Sor), 275 mM; and 5% Man, 275 mM. Twenty micrograms of total RNA, isolated from rosette leaves of more than seven plants for each treatment, were analyzed for the levels of β-amylase mRNA by northern blot hybridization. The levels of α-tubulin mRNA were similar in the various samples of RNA (data not shown). NT, Rosette leaves from nontreated plants.

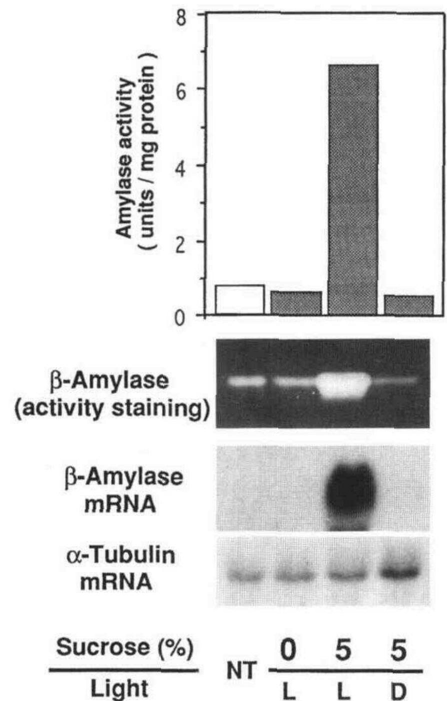

Figure 3. Effects of light on the induction of β-amylase by Suc. Three-week-old plants were treated with a solution of 5% Suc either under continuous light (L) or in darkness (D) for 3 d. The determination of total amylolytic activity, activity staining of β-amylase in the gel, and northern blot hybridization for quantitation of β-amylase and α-tubulin mRNAs was carried out as described in the legend to Figure 1. NT, Rosette leaves from nontreated plants.

Figure 2A shows the time course of increases in the level of mRNA for β-amylase in rosette leaves in response to treatment of plants with 5% Suc under continuous light. A significant increase in the level of mRNA for β-amylase was observed within 1 d of treatment with Suc. The amylolytic activity in rosette leaves also increased 2- to 4-fold within 1 d after the start of treatment with Suc and this activity continued to increase for 3 d (data not shown).

The mRNA for β-amylase was also induced when plants were treated with a 5% solution of Fru or Glc (Fig. 2B). However, neither a 5% solution of mannitol nor a 5% solution of sorbitol induced the accumulation of the mRNA for β-amylase. The results suggest that a specific carbohydrate-mediated metabolic signal rather than an osmotic signal is important for induction. Treatment of plants with a 5% solution of Glc or Fru, but not with a 5% solution of mannitol or sorbitol, also resulted in the accumulation of starch (data not shown).

The Sugar-Inducible Expression of the Gene for β-Amylase in *Arabidopsis* Requires Light

In sweet potato, the sugar-induced expression of genes that encode β-amylase and sporamin and the sugar-induced accumulation of starch in leaf-petiole explants occurs in darkness (Nakamura et al., 1991). When *Arabidopsis*

plants were cultured with a solution of 5% Suc in darkness for 3 d, neither an increase of the activity of β-amylase nor accumulation of the mRNA for β-amylase was observed (Fig. 3). Although dramatic effects of light were observed in many separate experiments, small increases in the levels of amylolytic activity and the mRNA for β-amylase occurred in the absence of light in some experiments. The effect of light may, therefore, depend on the physiological state of the plant.

As shown in Figure 4, the presence of 20 μM DCMU, an inhibitor of electron transport in PSII, did not inhibit the sugar-inducible expression of the gene for β-amylase under continuous light. Although DCMU is known to be easily taken up by plant tissues (Peña-Cortés et al., 1992), it might not have been taken up from the roots. However, results similar to those presented in Figure 4 were obtained when excised leaves were used (data not shown). The absence of inhibition by DCMU suggests that photosynthesis is not a component of the mechanism of induction. Chloramphenicol at 300 μM also had no effect on the induction of the mRNA for β-amylase. However, cycloheximide at 300 μM strongly blocked the accumulation of this mRNA after treatment with 5% Suc (Fig. 4). It is suggested that the sugar-inducible expression of the gene for β-amylase in the light requires the de novo synthesis of proteins in the cytoplasm.

Sugar-Inducible Expression of the Gene for β-Amylase in Various Organs of *Arabidopsis*

Extracts of various organs of *Arabidopsis* plants that had been treated with 5% Suc in the presence or absence of light for 3 d were assayed for the levels of amylolytic activity and β-amylase protein. As shown in Figure 5, extracts from stems, bracts (leaves attached to the stem), and roots of nontreated mature plants yielded a band of amylolytic activity that migrated at the same position as that of the β-amylase from rosette leaves. In addition to the results in rosette leaves, the levels of amylolytic activity and β-amylase protein in these organs increased significantly when plants were cultured with a solution of Suc under continuous light (Fig. 5). When Suc was supplied to plants in darkness, there was no significant increase in the β-amylase activity in these organs. Glc also induced increases in β-amylase activity in various organs (data not shown).

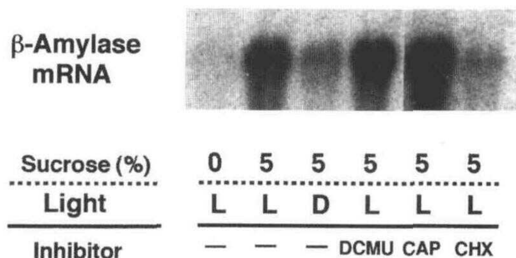

Figure 4. Effects of DCMU, cycloheximide, and chloramphenicol on the induction of β-amylase by Suc. Three-week-old plants were treated with a solution of 0 or 5% Suc either under continuous light (L) or in darkness (D) for 1 d. For treatment with inhibitors, plants were pretreated with 20 μM DCMU, 300 μM chloramphenicol (CAP), or 300 μM cycloheximide (CHX) for 2 h before treatment with a 5% solution of Suc that also contained the inhibitor. Twenty micrograms of total RNAs, isolated from rosette leaves of more than seven plants for each treatment, were analyzed to determine the level of β-amylase mRNA by northern blot hybridization. The levels of α-tubulin mRNA were similar in the various samples of RNA (data not shown).

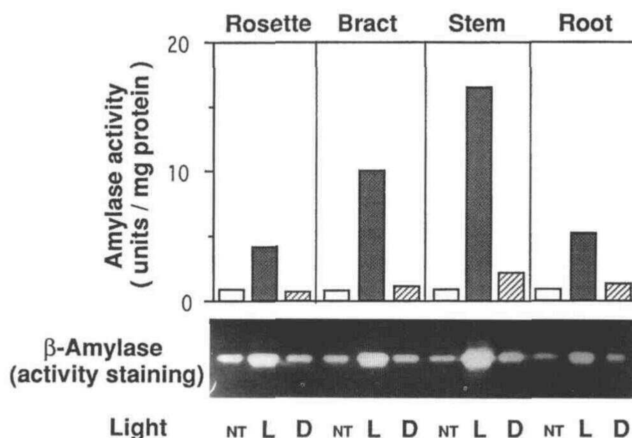

Figure 5. Induction of β-amylase in various organs of *Arabidopsis* by treatment with Suc. Roots of 3-week-old plants were dipped in a 5% solution of Suc either under continuous light (L) or in darkness (D) for 3 d. Determinations of total amylolytic activity and activity staining of β-amylase in the gel were performed. Similar results were obtained in two separate experiments. NT, Rosette leaves from nontreated plants.

Isolation of a Nuclear Gene for β-Amylase from *Arabidopsis*

Total genomic DNA from *Arabidopsis* plants was digested with the restriction enzymes *Sal*I, *Sst*I, *Xba*I, *Hin*dIII, *Xho*I, and *Pst*I and the Southern blot filters were hybridized with the ^{32}P-labeled fragment of the gene for β-amylase obtained by PCR. Only one band hybridized with the probe in each lane after hybridization under both low-stringency (15°C below the Tm; data not shown) and high-stringency conditions (5°C below the Tm; Fig. 6). These results suggest that the major β-amylase of *Arabidopsis* is encoded by a gene that is present as a single copy per haploid genome.

We screened a genomic library of *Arabidopsis* with the ^{32}P-labeled fragment from PCR, and we isolated three different positive clones. The inserts from these clones gave restriction maps that overlapped one another and yielded restriction fragments that were identical in size with fragments of genomic DNA detected in the Southern blot hybridization shown in Figure 6. The gene for β-amylase (*Bmy3*[2]; International Society of Plant Molecular Biology No. 259) in one of the phage clones, λATβA3, was characterized further.

The nucleotide sequence of about 4.8 kb that included the gene for β-amylase was determined. In the determined

[2] A nomenclature for sequenced plant genes (Plant Mol Biol Rep **11:** 291–316 [1993]). The nucleotide sequence reported in this paper had been submitted to the DDBJ data base under the accession number D43783.

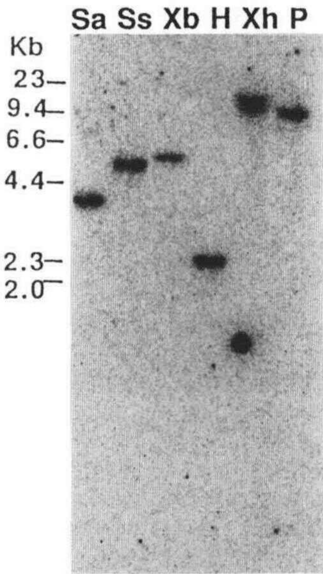

Figure 6. Southern blot analysis of *Arabidopsis* DNA. Total DNA from *Arabidopsis* plants was digested with *Sal*I (Sa), *Sst*I (Ss), *Xba*I (Xb), *Hin*dIII (H), *Xho*I (Xh), and *Pst*I (P). Hybridization of the membrane was carried out with a ^{32}P-labeled fragment from PCR that encoded part of β-amylase. The sizes of fragments are those of fragments of *Hin*dIII-digested λ phage DNA.

sequence, the sequence that corresponded to that of the cDNA was interrupted by six introns (Figs. 7 and 8). The nucleotide sequence of the exons contained an open reading frame of 1494 nucleotides that was identical with the sequence of the cDNA clone (Monroe et al., 1991) with the exception that a T residue at position 2694 of the cDNA was replaced by a G residue in our nucleotide sequence, a change that resulted in substitution of Leu for Phe. All of the introns conformed to the GT-AG rule for exon-intron borders (Fig. 7) and they were located within the coding region at exactly the same positions as introns in the gene for β-amylase from sweet potato (Yoshida et al., 1992; Fig. 8). The nucleotide sequence of the 3′ untranslated region of the genomic sequence downstream from +2789 (Fig. 7) differed from the corresponding region of the cDNA isolated by Monroe et al. (1991), and the genomic sequence contained several putative polyadenylation signals (Fig. 7) that were absent from the sequence of cDNA. It is not known whether the difference in nucleotide sequence is due to the difference in the ecotypes of plant that were used for isolation of the genomic clone (Landsberg *erecta*) and the cDNA clone (Columbia).

From the primer extension analysis of the 5′ terminus of the transcript of the gene for β-amylase, the residue that corresponded to the 5′ terminus of the extension product was taken as the site of the initiation of transcription and it was numbered +1 in the sequence shown in Figure 7. A few minor bands of extension products that were longer or shorter by one or two nucleotides were also detected (data not shown). A TATA box-like sequence, TATATAA, was found at a site 25 bp upstream from the potential site of initiation of transcription. Unlike the nucleotide sequence

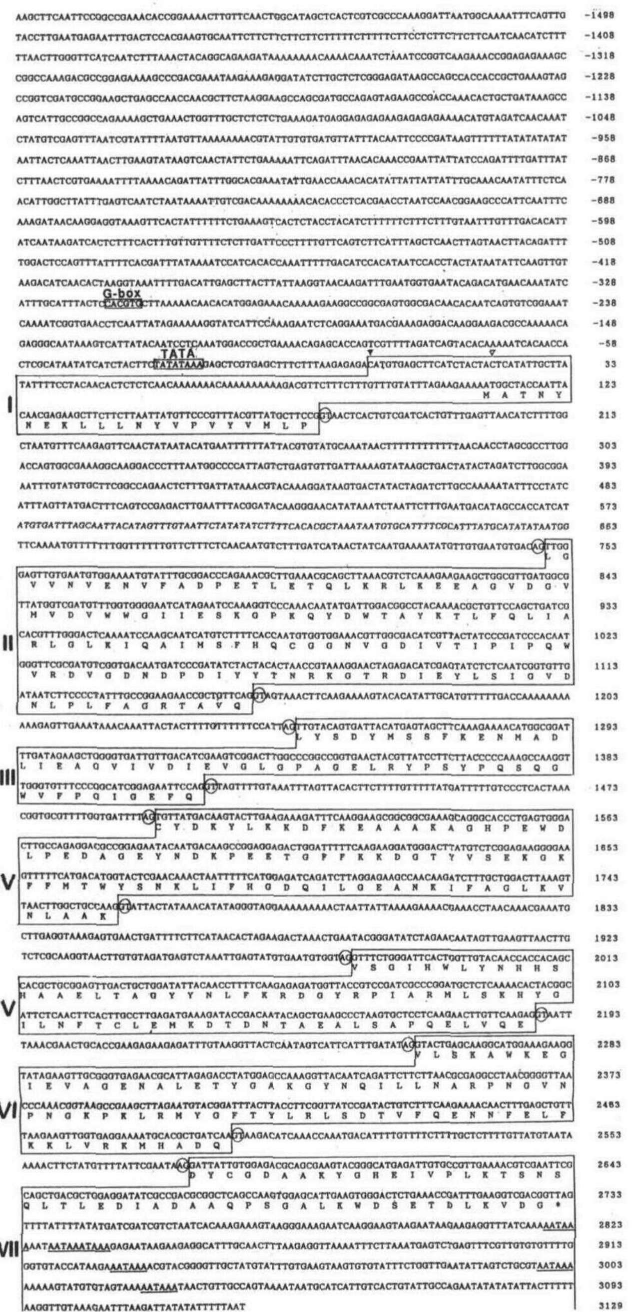

Figure 7. Nucleotide sequence of the gene for β-amylase of *Arabidopsis* and the deduced amino acid sequence. Nucleotides are numbered from the site of initiation of transcription (see text). Seven exon sequences, a putative TATA box, and a G-box motif are boxed. Nucleotides corresponding to the 5′ terminus of the major product of primer extension and the 5′ terminus of the cDNA reported by Monroe et al. (1991) are indicated by solid and open arrowhead, respectively. The dinucleotides GT and AG at the exon-intron boundaries are circled. The putative polyadenylation signals in the seventh exon are underlined. The amino acid sequence of β-amylase deduced from the nucleotide sequence is shown in the one-letter code beneath the first nucleotide of each respective codon.

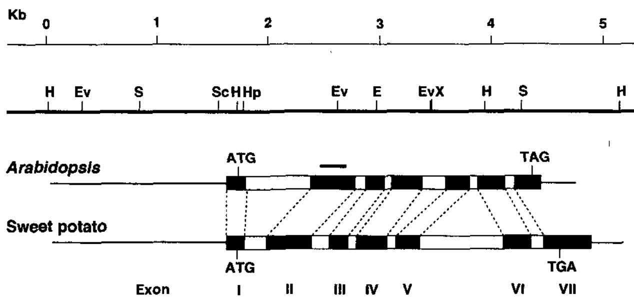

Figure 8. Comparison of exon-intron structures of genes for β-amylase of *Arabidopsis* and sweet potato. Top, Restriction endonuclease map of genomic DNA that corresponds to the gene for β-amylase of *Arabidopsis*. H, *Hin*dIII; Ev, *Eco*RV; S, *Sal*I; Sc, *Sac*I (*Sst*I); Hp, *Hpa*I; E, *Eco*RI; X, *Xba*I. Bottom, Exon-intron structure of the gene for β-amylase of *Arabidopsis* (Fig. 7) compared to that of the sweet potato gene (Yoshida et al., 1992). Solid and open boxes indicate exons and introns, respectively. Exons are numbered from I to VII. Positions of codons for the initiation and termination of translation are indicated. The bar shown above the second exon of the gene for β-amylase of *Arabidopsis* corresponds to the region that was amplified by PCR.

of the exons, the nucleotide sequences of the introns and the 5′ upstream region of the gene for β-amylase from *Arabidopsis* differed significantly from those of the gene for β-amylase from sweet potato (data not shown).

Sugar-Inducible Expression of the *ATβ-Amy:GUS* Fusion Gene in Transgenic *Arabidopsis*

We constructed a translational GUS fusion gene (*ATβ-Amy:GUS*) in which the sequence from −1587 to +136 of the gene for β-amylase from *Arabidopsis* was fused with the coding sequence of GUS (Fig. 9A), and we introduced this fusion gene into the genome of *Arabidopsis* by *Agrobacterium*-mediated transformation (Valvekens et al., 1988; Akama et al., 1992). The binary vector used for this purpose was constructed by inserting the CaMV 35S promoter: hygromycin phosphotransferase fusion gene downstream of the *ATβ-Amy:GUS* fusion gene in the opposite orientation for the selection of transformants by resistance to hygromycin (Akama et al., 1992). Insertion of the CaMV 35S promoter:hygromycin phosphotransferase fusion gene in the same orientation as the *ATβ-Amy:GUS* fusion gene resulted in unusually high levels of expression of GUS (data not shown).

Rosette leaves detached from 12 transgenic plants were treated with a solution of 6% Suc either under continuous light or in darkness for 1 d and the GUS activity in each extract was determined. Representative results for two independent transgenic lines with different levels of GUS activity are shown in Figure 9B. Results obtained with other transgenic lines were similar to those shown in Figure 9B. Treatment of leaves with Suc under continuous light resulted in significant enhancement of GUS activity, as well as of the activity of endogenous β-amylase. By contrast, treatment of leaves with Suc in darkness did not result in a significant increase in GUS activity. Treatment of leaves with a 6% solution of Glc or Fru, but not of sorbitol or mannitol, also caused increases in GUS activity (data not shown). Expression of an intron-GUS fusion gene driven by the CaMV 35S promoter (Ohta et al., 1990) in leaves of transgenic *Arabidopsis* (Akama et al., 1992) was not affected by the treatment with solutions of Suc under continuous light despite an increase in the activity of endogenous

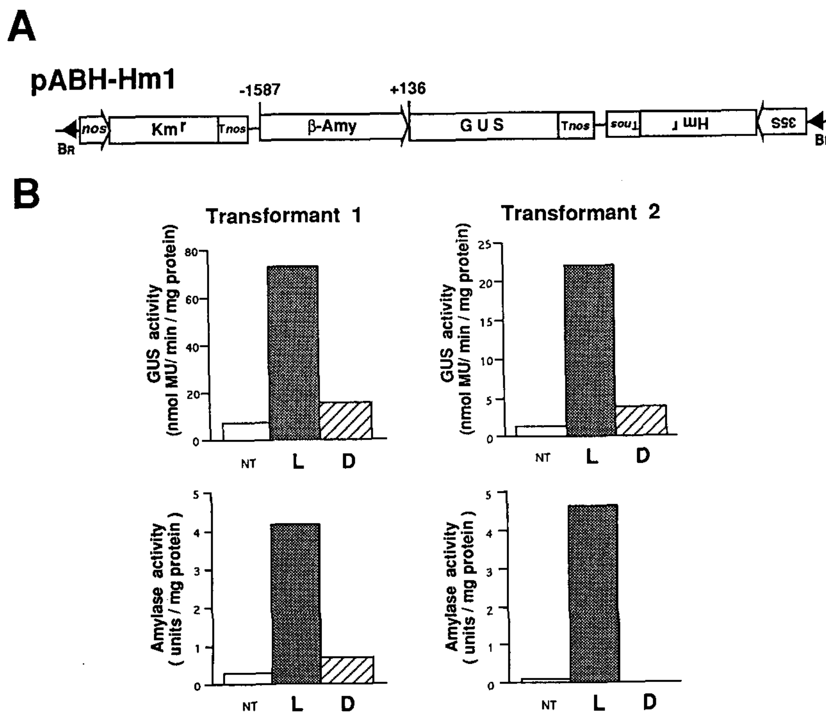

Figure 9. Suc-inducible expression of the *ATβ-Amy:GUS* gene fusion in leaves of transgenic *Arabidopsis*. A, Schematic representation of the structure of the T-DNA in a binary vector, pABH-Hm1, that contained the *ATβ-Amy:GUS* fusion gene. T*nos*, Terminator fragment of the *nos* gene; 35S, CaMV 35S gene promoter. T-DNA border sequences (B_R and B_L) are indicated by solid arrows. B, Rosette leaves detached from two independent transgenic lines (1 and 2) were treated with 6% Suc either under continuous light (L) or in darkness (D) for 1 d. Activities of both GUS (top) and endogenous β-amylase (bottom) in the extracts were determined. NT, Nontreated rosette leaves.

β-amylase (data not shown). These results suggest that the sugar-inducible expression of the gene for β-amylase of *Arabidopsis* in the light is regulated at the level of transcription, which is mediated by the 1.6-kb-long 5′ upstream region of the gene for β-amylase. However, a role for the nucleotide sequence from +1 to +136 in the regulation cannot be ruled out at present.

DISCUSSION

The β-amylases in the vegetative tissues of plants are most probably located outside the chloroplasts where the synthesis and accumulation of starch occurs (Beck and Ziegler, 1989) and the physiological roles of such β-amylases are not clearly understood. Caspar et al. (1989) reported previously that the level of β-amylase in rosette leaves of *Arabidopsis* was very high in certain mutants with altered starch metabolism under conditions associated with high-level accumulation of soluble sugars in leaves. The present study was aimed at obtaining a clearer picture of the regulation of expression of the gene for β-amylase in *Arabidopsis*.

The amylolytic activity in rosette leaves of *Arabidopsis* was significantly enhanced by an exogenous supply of large amounts of Suc to roots of intact plants (Fig. 1A) and to detached leaves (Fig. 9). This enhancement of amylolytic activity in rosette leaves occurred with a concomitant increase in the intensity of a band of protein with β-amylase activity and in the level of mRNA for β-amylase (Fig. 1). Expression of the gene for β-amylase was also induced by Glc and by Fru but not by mannitol or by sorbitol (Fig. 2B). The sugar-inducible expression of the gene for β-amylase occurred not only in rosette leaves but also in roots, stems, and bracts (Fig. 5). The results indicate that expression of the single-copy gene for β-amylase in *Arabidopsis* (Fig. 6) is inducible in various vegetative tissues by a carbohydrate-mediated metabolic signal, and they also suggest that the expression of the gene in various tissues may be regulated by the carbon partitioning and sink-source interactions in the whole plant.

The gene for β-amylase of sweet potato is also inducible by sugars. Although β-amylase is an abundant protein in the tuberous roots of sweet potato, it is absent or present only in small amounts in organs other than the tuberous roots (Nakamura et al., 1991; Yoshida and Nakamura, 1991). However, the enzyme can be induced to high levels in other vegetative tissues, such as stems, petioles, and leaves, by a supply of large amounts of Suc or other metabolizable sugars (Nakamura et al., 1991). The induction of β-amylase by sugars occurs concomitantly with the induction of sporamin, the most abundant protein in tuberous roots, and with the accumulation of starch. The sugar-induced accumulations of sporamin, β-amylase, and starch exhibit similar dependence on the concentration of Suc (Nakamura et al., 1991) and each occurs within the same cells in various tissues (Takeda et al., 1995). It is suggested that the accumulation of β-amylase in sweet potato is closely connected to the cellular expression of the functions necessary for storage of metabolites when an excess of usable carbon sources is available and that β-amylase may play in part a role of a storage protein (Nakamura et al., 1991). However, the amount of β-amylase that accumulates in Suc-treated leaves of *Arabidopsis* seems to be much smaller than that expected for a storage protein (data not shown). The β-amylase in cotyledons of mustard has been reported to be a stable protein with a low turnover rate (Subbaramaiah and Sharma, 1989). It seems likely that β-amylase is not synthesized solely as a vegetative storage protein.

Substrate(s) of extrachloroplastic β-amylases in vivo is unknown. The major β-amylase from pea epicotyls readily hydrolyzes maltodextrins with a relatively low degree of polymerization (Lizotte et al., 1990). Although it has been proposed that β-amylase plays a role in the hydrolysis of maltodextrins that are stored in the vacuoles, the presence of such α-glucans has not been proved (Beck and Ziegler, 1989; Lizotte et al., 1990). It is possible that such glucans might be synthesized specifically under the conditions that also result in induction of β-amylase, such as when an excess of usable carbon source is available, and β-amylase might rapidly hydrolyze such glucans to prevent their accumulation. In addition to its amylolytic activities, the β-amylase of sweet potato has been shown to be able to inhibit a starch phosphorylase in vitro (Pan et al., 1988). However, the physiological significance of this inhibitory activity in vivo is unknown. The extent of the sugar-inducible expression of the gene for β-amylase in *Arabidopsis* was greatly diminished in the absence of light, not only in rosette leaves (Figs. 3 and 9) but also in other tissues (Fig. 5). The effect of light may depend on the physiological conditions of plants since the sugar-inducible expression of the gene in darkness was observed at lower levels in some experiments (data not shown). Although a phytochrome-mediated increase in levels of β-amylase mRNA occurs in mustard cotyledons (Sharma and Schopfer, 1987), light alone does not induce the expression of the gene for β-amylase in *Arabidopsis*.

As shown in Figure 4, the sugar-inducible expression of the gene in the presence of light was not inhibited by DCMU, an inhibitor of the photosynthetic electron transport that is easily taken up by plant tissues (Peña-Cortés et al., 1992). Although a possibility of ineffective uptake of DCMU to plants or explants in our experiments remains, it is suggested that light might be required for a pathway other than the photosynthesis. The sugar-inducible expression of class I genes for patatin in detached leaves of potato also requires light, and the simultaneous supply of Gln with sugars can compensate the absence of light (Peña-Cortés et al., 1992). In our preliminary experiments, a supply of Gln or ammonium ions in addition to Suc only partially overcame the effects of the absence of light in the induction of the gene for β-amylase (data not shown). Further analyses are required to clarify the role of light in the sugar-inducible expression of this gene in *Arabidopsis*.

A number of genes that are inducible by elevated levels of sugars have been identified in various plant species. However, it is not known whether the sugar-inducible expression of various genes in plants is mediated by a common mechanism. In the case of sweet potato, multiple

genes coding for sporamin and the gene for β-amylase are coordinately regulated under various conditions (Hattori et al., 1990, 1991; Nakamura et al., 1991; Ohto et al., 1992; Takeda et al., 1995), and the gene for the small subunit of ADP-Glc pyrophosphorylase is also inducible by sugars (Takeda et al., 1994). The sugar-inducible expression of all of these genes in sweet potato is blocked by inhibitors of protein phosphatases 1 and 2A, namely, okadaic acid, microcystin-LR, and calyculin A (Takeda et al., 1994). By contrast, sugar-inducible expression of class I genes for patatin and genes for proteinase inhibitor II in leaves of potato are regulated differently with respect to their responses to light, to cycloheximide, and to chloramphenicol (Peña-Cortés et al., 1992). In *Arabidopsis*, the expression of several genes in addition to the gene for β-amylase has been reported previously to be inducible by sugars (Tsukaya et al., 1991; Cheng et al., 1992; Yang et al., 1993). The light-inducible expression of the *NR1* gene that encodes nitrate reductase in dark-adapted *Arabidopsis* plants can be mimicked by a supply of a 2% solution of Suc to plants in darkness, the results suggesting that light stimulates the expression of the *NR1* gene via a supply of sugars generated by photosynthesis (Cheng et al., 1992).

Expression of the *ATβ-Amy:GUS* fusion gene responded to sugars in a light-dependent manner, as did the endogenous gene for β-amylase in transgenic *Arabidopsis* plants (Fig. 9). The results suggest that the regulation occurs at the level of transcription, although the role of the nucleotide sequence from +1 to +136 in the regulation cannot be ruled out. The sugar-inducible expression of the gene for β-amylase in sweet potato occurs in the absence of light (Nakamura et al., 1991) and the 5′ upstream region of the gene from sweet potato confers the capability for sugar-inducible expression in darkness on the GUS reporter gene in leaves of transgenic tobacco (Takeda et al., 1994). The nucleotide sequence of the 5′ upstream region of the gene for β-amylase of *Arabidopsis* differs significantly from that of the sweet potato gene. The G-box motif sequence (Williams et al., 1992) is present in the genes for β-amylase of both sweet potato (Ishiguro et al., 1993) and *Arabidopsis* (Fig. 7). A common nuclear factor of sweet potato binds to sequence elements designated SP8 that is present in multiple locations in the 5′ upstream regions of genes for sporamin and β-amylase of sweet potato (Ishiguro and Nakamura, 1992, 1994). Although the SP8 sequence motif may not be directly involved in the sugar-inducible expression of the sporamin-GUS fusion gene in leaves of transgenic tobacco (A. Morikami, S. Ishiguro, and K. Nakamura, unpublished results), sequences similar to the SP8 motif are suggested to be involved in the sugar-responsive expression of a class I patatin gene in potato (Grierson et al., 1994). The SP8 motif sequence is not present in the 5′ upstream region of the gene for β-amylase of *Arabidopsis*. Analysis of the *cis* regulatory sequences that are involved in the light-dependent and sugar-responsive expression of the gene for β-amylase of *Arabidopsis* is in progress.

The amylolytic activity in leaves of *Arabidopsis* can be attributed for the most part to β-amylase and can easily be assayed with soluble starch and staining with I_2/KI. Taking advantage of these features, we can easily screen mutants of *Arabidopsis* for altered expression of the gene for β-amylase. Such mutants should help us to understand the molecular mechanisms responsible for the sugar-inducible expression of the gene for β-amylase and the physiological role of the enzyme. Characterization of mutants of *Arabidopsis* in which expression of the gene for β-amylase exhibits altered response to sugars will be described elsewhere.

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