Sugar Response Sequence in the Promoter of a Rice α-Amylase Gene Serves as a Transcriptional Enhancer*

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Expression of α-amylase genes in both rice suspension cells and germinating embryos is repressed by sugars and the mechanism involves transcriptional regulation. The promoter of a rice α-amylase gene AerAmy3 was analyzed by both loss- and gain-of-function studies and the major sugar response sequence (SRS) was located between 186 and 82 base pairs upstream of the transcription start site. The SRS conferred sugar responsiveness to a minimal promoter in an orientation-independent manner. It also converted a sugar-insensitive rice actin gene promoter into a sugar-sensitive promoter in a dose-dependent manner. Linker-scan mutation studies identified three essential motifs: the GC box, the G box, and the TATCCA element, within the SRS. Sequences containing either the GC box plus G box or the TATCCA element each mediated sugar response, however, they acted synergistically to give a high level glucose starvation-induced expression. Nuclear proteins from rice suspension cells binding to the TATCCA element in a sequence-specific and sugar-dependent manner were identified. The TATCCA element is also an important component of the gibberellin response complex of the α-amylase genes in germinating cereal grains, suggesting that the regulation of α-amylase gene expression by sugar and hormone signals may share common regulatory machinery.

Sugar repression of gene expression is a fundamental and ubiquitous regulatory system for adjusting to changes in nutrient availability in both prokaryotic and eukaryotic cells. In microorganisms, glucose or other rapidly metabolizable carbon sources repress the expression of genes that code for enzymes related to the metabolism of other carbon sources. Our understanding of the mechanisms of sugar repression has been based largely on studies of microorganisms. In the case of Escherichia coli, a model to explain at the molecular level, the mechanism of sugar repression has been determined (1, 2). The mechanism of glucose repression in yeast is more complicated and is less understood than it is in E. coli (3–5). Studies using Saccharomyces cerevisiae mutants have revealed many of the components involved in the response to carbon catabolite repression (5), but it is still unclear how all of these components interact to regulate transcription. A universal signaling pathway which leads to the regulation of all glucose-repressible genes has yet to be determined.

As in microorganisms, sugar repression of gene expression also allows plant cells to cope effectively with changes in the carbon sources present in their environment. However, in multicellular plants, feedback repression by excess sugars provides an additional mechanism for maintaining an economical balance between supply (source) and demand (sink) for carbohydrate allocation and utilization among tissues and organs (6–8). Despite the fact that sugar repression of gene expression is likely a central control mechanism mediating energy homeostasis and carbohydrate distribution in plants, the molecular mechanism of sugar feedback regulation remains elusive. For example, sugar feedback regulation of genes involved in photosynthesis (9) and carbohydrate metabolism (10–12) has been shown to act at the level of gene transcription. A conserved glucose-sensing mechanism, via the action of hexokinase, has been observed between plants and microorganisms (13–15). However, the mechanism which connects the sensing and transmission of sugar signals and the repression of gene transcription in plants is mostly unknown.

The sugar-dependent repression of α-amylase gene expression provides an ideal model for studies on the molecular mechanisms that mediate glucose repression in plants. α-Amylases are endo-amylolytic enzymes which catalyze the hydrolysis of α,1,4-linked glucose polymers that play an important role in the degradation of starch and glycogen in higher plants, animals, and many microorganisms. α-Amylases in plants are recognized as essential enzymes whose major function is hydrolysis of starch stored in the endosperm during germination of cereal grains. Expression of α-amylase genes in rice is found under different modes of tissue-specific regulation: in the embryo of germinating seeds and in cultured suspension cells, expression is activated by sugar deprivation and repressed by sugar provision (8, 16–18); in the endosperm of germinating seeds, expression is activated by gibberellic acid and repressed by abscisic acid and osmotic stress (8, 19). Studies with rice suspension cells have shown that α-amylase expression, carbohydrate metabolism, and vacuolar autophagy are coordinately regulated by sucrose levels in the medium (20). Both the transcription rate and mRNA stability of α-amylase genes in cells increase in response to sucrose depletion in the culture medium (12). Use of transgenic rice carrying an α-amylase gene promoter-β-glucuronidase (GUS)† gene proved that the regulation

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1 The abbreviations used are: GUS, β-glucuronidase; bp, base pair(s); PCR, polymerase chain reaction; CaMV35S, cauliflower mosaic virus 35S RNA; MES, 4-morpholineethanesulfonic acid; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; SRS, sugar response sequence; Luc, luciferase.
of α-amylase gene expression by sugars involves a transcriptional control mechanism (10, 21, 22). Sugar-dependent repression of α-amylase gene expression has also been observed in Aspergillus oryzae (23) and Drosophila melanogaster (24) and the mechanism was shown to involve transcriptional control (25, 26).

Rice α-amylase isozymes are encoded by at least nine genes (7). By using an α-amylase gene-specific DNA fragments and nuclear run-on transcription analysis, transcription of eight α-amylase genes was shown to increase in response to sucrose starvation (27). A positive correlation between the transcription rates and the steady-state mRNA levels suggests that transcriptional regulation plays an important role in the differential expression of individual α-amylase genes. To date, studies on the transcriptional regulation of α-amylase gene expression in plants have mostly focused on the hormonal regulation in germinating cereal grains (28–33). Since sugars regulate the expression of α-amylase genes in germinating seeds as well as in cultured suspension cells, studies on the mechanism of sugar feedback regulation using rice suspension cells as a model system may lead us to a better understanding of the mechanisms controlling carbohydrate metabolism in higher plants. We chose αAmy3 as a model gene for this study because it constitutes approximately 60% of total α-amylase mRNAs in cells starved for sucrose (27) and its expression in germinating embryo is regulated by sugars (8, 16). The αAmy3 promoter has been shown to mediate sugar-dependent regulation of GUS reporter gene expression in transgenic rice suspension cells (22).

In this report we developed a transient expression system using protoplasts prepared from rice suspension cells to determine the cis-acting sugar-responsive sequences in the αAmy3 promoter. The literature has revealed that, in the case of studying regulation by environmental and physiological cues, results obtained from transient assays often rapidly and efficiently provide important information for further studies and reflect the in vivo situation in planta. For example, studies on auxin (34, 35) and gibberellin (28, 29, 32) response elements using transient assays have led to the identification of interacting transcription factors (28, 36). By conducting loss-of-function, gain-of-function, and linker-scan mutation analyses, we defined the minimal sequence in the αAmy3 promoter that drives high level glucose starvation-induced expression. This minimal sequence served as a transcriptional enhancer and converted a sugar-insensitive promoter into a sugar-sensitive promoter. We also identified three essential motifs, the GC box, the G box, and the TATCCA element, that form the sugar response complex and act cooperatively in controlling αAmy3 expression. Finally, we demonstrated that nuclear proteins from rice suspension cells bind to the TATCCA element in a sequence-specific and sugar-dependent manner. To our knowledge, this is the first identification of the functional sugar response elements and the existence of interacting trans-acting factors for sugar repressible genes in plants.

**EXPERIMENTAL PROCEDURES**

**Rice Cell Culture—**Suspension cell cultures of rice (*Oryza sativa* cv. Tainan 5) were propagated as described previously (17). Established suspension cells were subcultured every 7 days by transferring about 0.5 ml of cells into 25 ml of fresh liquid Murashige and Skoog medium (37) containing 3% sucrose in a 125-ml flask. Cells were cultured on a rotary shaker at 120 rpm and incubated at 26 °C in the dark.

**Primer Extension Analysis—**Three 18–20-base gene-specific oligonucleotides complementary to the signal peptide regions of αAmy7 and αAmy8, and the 5′-untranslated leader of αAmy3 (Fig. 1B) were synthesized and used as primers. The primer extension analysis was performed according to Sutliff et al. (38).

**Plasmid Construction—**A 1.7-kilobase SalI-EcoNI fragment, containing the promoter, the 5′-untranslated sequence, and 84 bp downstream of the translation start site of αAmy3 was end-blunted and cloned into the ClaI site of pBSI-132, forming p3G-132II. pBSI-132 was generated by insertion of a PvuII fragment (GUS coding sequence-nopaline synthase gene (nos) terminator fusion) from pBSI (21) into the HindIII site of pTRA132 (cauliflower mosaic virus 35S RNA (CaMV35S) promoter-h) coding a tobacco tumor morphology large gene (tm1) terminator fusion) (39) by blunt-end ligation. Plasmid p3G-132II was used as the progenitor for all constructs reported in this paper. The sequences of oligonucleotides used in preparation of the αAmy3 promoter constructs are listed in Table 1. Appropriate combinations of 5′ and 3′ primers were used to generate different 5′ deletions, internal deletions, and other mutations by polymerase chain reaction (PCR). Series of 5′ and 3′ end deletion constructs were digested with EcoRI and PstI and ligated into pBluescript K5+ (Stratagene) to generate p3.4, p3.5, and p3.6. These plasmids were digested with XhoI and PstI and the promoter regions were ligated into the KpnI and PstI-digested pLuc, forming p3Luc.3, p3Luc.4, p3Luc.5, and p3Luc.6. pLuc was generated by insertion of a SalI-BglII fragment (luciferase (Luc) coding sequence-nos terminator fusion) (from pJDS121) into the StuI site of pBluescript K5+ (Stratagene) to generate p3Luc.7, p3Luc.8, and p3Luc.9.

To prepare constructs for the gain-of-function analysis, a CaMV35S minimal promoter-alcohol dehydrogenase intron I (AdhI) fragment was obtained by PCR using pJD312 as template, inserted into the PstI and NcoI sites of pLuc to generate p35mALuc. DNA fragments containing various regions of the wild type or mutant αAmy3 promoters were PCR amplified, cloned into pBluescript, and generated p3.15 through p3.19 and p3.40. These plasmids were digested with XhoI and PstI and the promoter regions were cloned into p35mALuc to generate p3Luc.15 through p3Luc.19 and p3Luc.40. Then p3Luc.18 containing SRS (–186 to –82) in correct orientation was digested with XhoI and PstI, blunt-ended, and religated. Plasmid containing SRS in reverse orientation was then generated and designated as p3Luc.18R. The –186 to –122 fragment in p3Luc.19 and the –132 to –82 fragment in p3Luc.40 were digested with PstI and XhoI and religated. Plasmids containing multiple deletion constructs, PCR was performed using the oligonucleotide-directed mutagenesis as described by Picard et al. (41). In this method, the 5′ internal deletion primers were first paired with 3′ primers to generate short DNA fragments. The PCR products then served as 5′ primers and paired with 3′ primers to generate internal deletion fragments. These fragments were cloned into pLuc using the same procedure for 5′-deleted constructs and generates p3Luc.7, p3Luc.8, and p3Luc.9.

For construction of plasmids carrying SRS in the ActI promoter, the ActI 5′ region (including 1.4-kb 5′-flanking sequence, 79-bp 5′ noncoding exon, 447-bp 5′ intron, and 225-bp first coding exon) was excised from pDM302 (42) with HindIII and subcloned into pBluescript. The EcoRI site in the multiple cloning sites of pBluescript was removed by digestion with EcoRV and XhoI and then blunt-ended and religated. The SRS sequence along with the 35S minimal promoter and part of the AdhI intron were excised with HindIII from p3Luc.18 and inserted into the HindIII site of pBluescript and generated p3Luc.18R. The SRS was excised with EcoRI from p3Luc.18R and inserted into the EcoRI site (–498) of ActI promoter in pBluescript in one, two, or three copies and generated p3Luc.37++, p3Luc.37++, and p3Luc.37+++. The three plasmids were then digested with SalI and PstI and the SRS-containing ActI promoter constructs were used to replace the 35S promoter and AdhI intron in pJD312 and generated p3Luc.37++, p3Luc.37+++, and p3Luc.37++++. The three plasmids were then digested with SalI and PstI and the SRS-containing ActI promoter constructs were used to replace the 35S promoter and AdhI intron in pJD312 and generated p3Luc.37++, p3Luc.37+++, and p3Luc.37++++. The three plasmids were then digested with SalI and PstI and the SRS-containing ActI promoter constructs were used to replace the 35S promoter and AdhI intron in pJD312 and generated p3Luc.37++, p3Luc.37+++, and p3Luc.37++++.
Inc.), washed three times with CPW7.4 buffer by centrifugation at 100 \( \times \) g for 5 min, and gently resuspended in 2 ml of CPW7.4 buffer. The protoplasts were layered on 5 ml of 0.6 M sucrose cushion in centrifugation for 10 s at 12,000 g. Protoplasts at the top of sucrose cushion were collected and transferred to 10 ml of CPW7.4 buffer. Cells were resuspended in 0.3 ml of extraction buffer and placed on ice for 10 min. Electroporation was performed with a tobacco variety Nicotiana tabacum L. cv. Petit Havana SR1 was used in this study.

| Oligonucleotide | Sequence | Position | Constructs |
|-----------------|----------|----------|------------|
| a | ATCTTCAACCACTGTGCTA | -987 ~ -968 | p3Luc.3, p3AH, p3Luc.7 |
| b | GTGGATAAGATGCTCATG | -450 ~ -432 | p3Luc.8, p3Luc.9 |
| c | AAATGGCTCCTGTTATCCA | -274 ~ -256 | p3Luc.4 |
| d | ATTATTTGTCCTGCTCTCT | -100 ~ -82 | p3Luc.5, p3Luc.15, p3Luc.16 |
| e | ATCGTGTTAAGGCTATGGA | +91 ~ +71 | p3Luc.17, p3Luc.20, p3Luc.21 |
| f | GTCCGCTGATGTTGCTCTCATATA | -181 ~ -172 and -123 ~ -109 | p3Luc.23, p3Luc.26 |
| g | GCCATGCTCTTCGCTCATTCT | -133 ~ -124 and -84 ~ -70 | p3Luc.7 |
| h | TGTCGTGCTGCCGCTTGTATATAT | -94 ~ -85 and -41 ~ -27 | p3Luc.8 |
| i | ATCCGCGCTGCTGTTAGA | -186 ~ -169 | p3Luc.9 |
| j | AGAGAGCCACACAAATAAT | -82 ~ -120 | p3Luc.10 |
| k | CCCCGGCCAGCTCCTTATCTCCTTC | -122 ~ -139 | p3Luc.11 |
| l | CCGTCGCTGGGTGCTTCTGCTTCT | -237 ~ -266 | p3Luc.12 |
| m | GCCCTGCGGTCTCTGCTCT | -210 ~ -239 | p3Luc.13 |
| n | AACGCCGCTGACTATGCTCCTGCTCCTC | -164 ~ -203 | p3Luc.14 |
| o | CCGGCGCGTTATCAGCTCTTGAAGC | -150 ~ -179 | p3Luc.15 |
| p | TATTTCTTCTGATCCCGCTCATTAT | -124 ~ -95 | p3Luc.16 |
| q | CCCGGCTCCGCAAGCTTACTGCGGGCC | -164 ~ -193 | p3Luc.17 |
| r | CGGGTCGCTGGGTGCTTCTGCTTCTC | -154 ~ -183 | p3Luc.18 |
| s | CCCGGCTCCGCAAGCTTACTGCGGGCC | -144 ~ -173 | p3Luc.24 |

**Electroporation and Protoplast Culture**—Plasmid DNA was transfected into rice protoplasts by electroporation. Each sample containing 2 \( \times \) 10^6 protoplasts in 0.4 ml of electroporation buffer was mixed with 20 \( \mu \)g of test plasmid DNA, 5 \( \mu \g of control plasmid DNA, and 50 \( \mu \)g of carrier (calf thymus) DNA. The mixture was transferred to a cuvette and placed on ice for 10 min. Electroporation was performed with an electroporator (BTX) with conditions set at 1000 V/cm, 400 microfarads, and 186 \( \Omega \). After electroporation, the protoplasts were kept on ice for 10 min, then mixed with 0.4 ml of electroporation buffer and 0.8 ml of 2 \( \times \) modified Murashige and Skoog medium (containing 0.2 mg of 2,4-dichlorophenoxyacetic acid and 0.1 mg of kinetin per liter) plus 400 mM glucose or 400 mM mannitol and 5 mM glucose. The protoplasts were plated in a 3-cm Petri dish and cultured 18 h at 26 °C in the dark.

**GUS and Luciferase Assays**—Protoplasts (2 \( \times \) 10^6) were collected by centrifugation for 10 s at 12,000 \( \times \) g, resuspended in 0.3 ml of extraction buffer (100 mM K\_2PO\_4, pH 7.8, 1 mM EDTA, 7 mM \( \beta \)-mercaptoethanol, 1% Triton X-100, and 10% glycerol), and vortexed for 15 s at high speed. The disrupted protoplasts were centrifuged at 12,000 \( \times \) g and 4 °C for 5 min. Supernatant was collected and used for GUS or luciferase activity assay. The enzyme activities in cell extract could be maintained stable for at least 1 month at -80 °C.

The fluorogenic assay for GUS activity was performed with modification of a method described by Jefferson (45). For each assay, 100 \( \mu \)l of 2 \( \times \) GUS assay buffer (100 mM NaPO\_4, pH 7.0, 20 mM \( \beta \)-mercaptoethanol, 20 mM Na\_2EDTA, 0.2% (w/v) sodium lauryl sarcosine, 0.25% (v/v) Triton X-100, and 1.8 mM 4-methylumbelliferyl \( \beta \)-D-glucuronide) was dispensed into a 1.5-ml Eppendorf tube. One hundred \( \mu \)l of cell extract was added and incubated at 37 °C in the dark for various lengths of time. Fifty \( \mu \)l of the reaction mixture was dispensed into 1950 \( \mu \)l of 0.2 M Na\_2CO\_3 immediately (\( t = 0 \) min) and repeated after 120 min. Fluorescence (excitation at 365 nm and emission at 455 nm) was determined using a TKO 100 fluorometer (Hoefer).

For luciferase assay, 50 \( \mu \)l of cell extract was placed in a luminometer cuvette (Starstedt), and then 180 \( \mu \)l of luciferase assay buffer (25 mM Tricine, pH 7.8, 15 mM potassium phosphate, pH 7.8, 15 mM MgSO\_4, 4 mM EGTA, 2 mM ATP, and 1 mM dithiothreitol) was added. The mixture was allowed to equilibrate to room temperature (about 15 min). Placing the cuvette in the counting chamber of a luminometer (LUMAT, Berthold) automatically activated the machine and 50 \( \mu \)l of 250 \( \mu \)l luciferin (Promega) was injected into the cuvette to start the reaction. The photons emitted were integrated over a 20-s period and expressed as relative light units/20 s.

Plasmid pUGI containing the ubiquitin promoter-GUS gene fusion served as an internal standard. pUGI was generated by insertion of the BanHI-HindIII fragment (ubiquitin (\( \beta \)-ubiquitin) promoter) from pAHCI8 (46) into pBSI digested with the same enzymes. Expression from the ubiquitin promoter was reduced less than 2-fold by glucose starvation of protoplasts. The GUS activity expressed from pUGI was used to standardize luciferase activity in cell extracts from cells grown with or without glucose.
Sugar Regulation of α-Amylase Gene

10123

Transgenic tobacco cell lines were obtained by transformation of leaf discs with Agrobacterium according to the method of Horsch et al. (48). Suspension cell cultures of the transgenic tobacco were propagated as described previously (17).

Preparation of Nuclear Extract—About 5 g (fresh weight) of rice suspension cells grown in the presence or absence of sucrose were pulverized in liquid nitrogen and homogenized in 200 ml of homogenization buffer (400 mM mannitol, 50 mM Tris-HCl, pH 7.9, 5 mM MgCl₂, 1 mM EDTA, 0.1% bovine serum albumin, 0.1% Nonidet P-40, 5 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride). After this step, preparation of nuclear extract followed the procedures as described by Mitsuana et al. (49).

Gel Mobility Shift Assay—Oligonucleotides F1 through F5 were synthesized and their sequences are shown in Fig. 1A. F2 used as probe was prepared by phosphorylation of the 5'-hydroxyl terminal with T4 polynucleotide kinase and [γ-32P]ATP (5000 Ci/mmol). DNA-protein binding reaction was carried out by incubation of 0.02 ng of labeled F2 with 20 μg of nuclear extract in a total volume of 20 μl of solution containing 17 mM Hapes, pH 7.9, 60 mM KCl, 7.5 mM MgCl₂, 0.12 mM EDTA, 17% glycerol, and 1.2 mM dithiothreitol, 0.5 μg of poly[d(C-D)] (Pharmacia), and 3 or 10 ng (150- or 500-fold amount of probe, respectively) competitor DNA. The assay mixture was incubated for 20 min at room temperature. After this step, electrophoresis of the assay mixture and autoradiography of gel followed the procedures as described by Mitsuana et al. (49).

RESULTS

Sequence Analyses of the α-Amylase Gene Promoters—Although the promoter regions of αAmy3(RAmy3D) (22), αAmy7(RAmy1A) (19), and αAmy8(RAmy3E) (10, 21) have been published (57). The sequences are numbered relative to the transcription start site (+1). The transcription start sites of three α-amylase genes have been mapped by primer extension analysis. The sequence complementary to the oligonucleotide primer used for primer extension is indicated by arrow. B, nucleotide sequences of the conserved regions in three α-amylase genes. Sequences of the promoter regions of αAmy3(RAmy3D), αAmy7(RAmy1A), and αAmy8(RAmy3E) have been published (57). The sequences are numbered relative to the transcription start site (+1). Thin head arrows indicate identical repetitive sequences.

Fig. 1. Nucleotide sequence comparison of the promoter regions of three rice α-amylase genes. A, alignment of sequences surrounding the TATA box and transcription start site regions (shown in bold). The sequences are numbered relative to the transcription start site (+1). The transcription start sites of three α-amylase genes were mapped by primer extension analysis. The sequence complementary to the oligonucleotide primer used for primer extension is indicated by arrow. B, nucleotide sequences of the conserved regions in three α-amylase genes. Sequences of the promoter regions of αAmy3(RAmy3D), αAmy7(RAmy1A), and αAmy8(RAmy3E) have been published (57). The sequences are numbered relative to the transcription start site (+1). Thin head arrows indicate identical repetitive sequences.

Fragment containing the 274-bp αAmy3 promoter-Luc in p3Luc.5 and the 100-bp αAmy3 promoter-Luc in p3Luc.6 were subcloned into binary vectors and transferred into tobacco via the Agrobacterium-mediated transformation. Ten independent transgenic tobacco plants for each construct were used for generation of suspension cell cultures and expression of luciferase was analyzed. As shown in Fig. 2B, the 274-bp promoter (pASLuc.5) conferred glucose starvation-induced expression of luciferase, whereas the 100-bp promoter (pASLuc.6) abolished the expression regardless of the concentration of glucose. Results of Fig. 2A and B, suggest that the cis-element(s) required for sugar-dependent regulation is located within the region between positions −274 and −100 of the αAmy3 promoter.

Because the conserved GC box, G box, and TATCCA element were located between −172 and −105 of the αAmy3 promoter (Fig. 1B), three constructs containing internal deletions between −174 and −42 were made (Fig. 2A). Deletions from −174 to −126 (including the GC box and G box) (pASLuc.7) or from −125 to −86 (including the TATCCA element) (pASLuc.8) led to a drastic decrease in the absolute level of luciferase activity but still conferred glucose response. Surprisingly, deletion from −85 to −42 (pASLuc.9) restored a glucose-dependent expression produced a dramatic drop in the level of glucose starvation-induced expression. Expression was further reduced when deletion was made to −274 (pASLuc.5). Despite the dramatic reduction in the absolute level of expression caused by the two promoter deletions, the fold induction of expression by glucose starvation was maintained at a similar level. Deletion of the next 174 bp (to position −100) (p3Luc.6) abolished the expression regardless of the concentration of glucose.

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similar to that of the full-length promoter (p3Luc.3). The above deletion analyses indicate that the regions between \(-990\) and \(-450\) and \(-274\) and \(-86\) are required for high level glucose starvation-induced expression of the \(\alpha\)Amy3 promoter.

**Functional Analysis of the Sugar Response Sequence in the \(\alpha\)Amy3 Promoter**—To determine whether the cis-acting element(s) required for sugar-dependent regulation is located within the region downstream of \(-274\) of the \(\alpha\)Amy3 promoter, fragments covering various regions between \(-274\) and \(-82\) were inserted upstream of a CaMV35S minimal promoter-AdhI-Luc fusion gene, as shown in Fig. 3. These constructs were then tested for transcriptional activity in rice protoplasts. Expression of the basic construct containing no \(\alpha\)Amy3 promoter sequence (p35mALuc) did not respond to glucose starvation. When the \(-274\) to \(-82\) (p3Luc.15) and \(-186\) to \(-82\) (p3Luc.18) fragments were fused upstream of the 35S minimal promoter, high levels of glucose starvation-induced expression of luciferase were observed. Fragment \(-274\) to \(-176\) contains three protein binding sequences, designated Box 1, Box 2, and Box 3 (49). Each of the three boxes contain a conserved GC-G(C/G) motif and have been proposed to be involved in sugar-dependent regulation of \(\alpha\)Amy3 promoter (49). Deletion of the promoter region containing the three boxes (p3Luc.18) resulted in a 30% increase of glucose starvation-induced expression, suggesting that this region may contain negative cis-acting elements. Surprisingly, when fragment \(-186\) to \(-82\) was inserted in reverse orientation upstream of the 35S minimal promoter (p3Luc.18R), expression of luciferase in response to glucose starvation was as high as that with the promoter fragment inserted in the correct orientation (p3Luc.18). These results demonstrate that the region between \(-186\) and \(-82\) contains most, if not all, of the cis-acting element(s) required to confer high level glucose starvation-induced expression on the 35S minimal promoter. We have designated this region as a sugar-response sequence (SRS).

**FIG. 2.** Deletion analysis identified two regions important for high level sugar-dependent expression of the \(\alpha\)Amy3 promoter. A, transient assay of luciferase activity in rice protoplasts. Plasmids carrying truncated \(\alpha\)Amy3 promoters were constructed by PCR as described under "Experimental Procedures." Each of these plasmids was co-transfected with pUGI into rice protoplasts. The transfected protoplasts were divided into two groups and cultured in medium containing 400 mM glucose or 5 mM glucose plus 400 mM mannitol. Luciferase and GUS activities were determined after 18 h. Error bars indicate the S.E. of three replicates for each construct. X indicates fold increase. B, assay of luciferase activity in transgenic tobacco suspension cells. Transgenic tobacco suspension cells carrying the 274-bp \(\alpha\)Amy3 promoter-Luc-Nos gene (derived from pA3Luc.5) and the 100-bp \(\alpha\)Amy3 promoter-Luc-Nos gene (derived from pA3Luc.6) were grown in the presence or absence of sucrose for 2 days. Cells were collected and luciferase activity was determined as described under "Experimental Procedures." Error bars indicate the S.E. of luciferase activity from 10 independent transgenic cell lines for each construct.
fragment −186 to −122 (p3Luc.19 × 2) increased the expression in response to glucose starvation. One copy of fragment −133 to −82 (p3Luc.40), which contains the duplicated TATCCA element, slightly elevated the expression of luciferase as compared with the control (p35mALuc), but no glucose response was observed. Interestingly, when this fragment was repeated in tandem (p3Luc.41), glucose response was restored and the absolute level of luciferase activity was higher than that of the promoter containing the GC box and G box (p3Luc.19 or p3Luc.19x2). To examine whether the sequence and/or position of TATCCA element relative to TATA box is important, mutations in the duplicated TATCCA elements were generated. Results showed that mutation in either the downstream (p3Luc.41m1) or the upstream (p3Luc.41m2) copy of the duplicated TATCCA element reduced the expression.

A 52-bp Fragment Containing the TATCCA Element Enhances Transcriptional Activity—Comparison of the luciferase activity produced by p3Luc.40 and p3Luc.41 in Fig. 3 suggests that the 52-bp fragment encompassing −133 to −82 enhances transcription. Mutation in the TATCCA element within this fragment reduced transcription, which further suggests that the TATCCA element is essential for enhancing transcription. To demonstrate the function in enhancing transcription, multiple copies of the 52-bp fragment were fused upstream of the CaMV35S minimal promoter and the luciferase activity was assayed. As shown in Fig. 4, duplication of the 52-bp fragment resulted in the increase of starvation induced luciferase activity. The increase became almost linear as more copies of the fragment were added. Luciferase activity in non-starved cells also increased linearly with additional copies of the 52-bp fragment, consequently, the fold induction of the luciferase activity by glucose starvation was not increased in parallel. The results suggest that the 52-bp fragment enhances transcription of the minimal promoter regardless of the glucose concentration.

SRS Acts as a Transcriptional Enhancer in a Sugar-insensitive Promoter—The SRS conferred high level glucose starvation-induced expression on the 35S minimal promoter in an orientation-independent manner, suggesting it may also function as a transcriptional enhancer. To confirm the enhancer function, SRS was inserted in one, two, and three tandem copies in the EcoRI site (∼459 bp upstream of the transcription start site) of the rice Act1 promoter (52). The wild type and the SRS-containing Act1 promoters were fused upstream of the Luc gene as shown in Fig. 5. These sugar response sequence constructs were then tested for transcriptional activity in rice protoplasts. Expression of the control construct (p35mALuc) was not detected. Expression of the wild type Act1 promoter (pActLuc) and the Act1 promoter containing one to three copies of SRS was similar in high concentration glucose. Expression of the Act1 promoter containing one copy of SRS (p3Luc.37) increased 2-fold as compared with that of the wild type Act1 promoter in the glucose-starved cells. Surprisingly, duplication of SRS (p3Luc.37++) dramatically increased fold induction by glucose starvation and the fold induction increased almost linearly as more copies of SRS were added (p3Luc.37+++). The results demonstrate that expression of the Act1 promoter becomes inducible by glucose starvation if the promoter is inserted with multiple copies of SRS.

Linker-scan Mutation Analysis of SRS in the αAmy3 Promoter—Understanding that SRS confers a high level glucose starvation-induced expression on the 35S minimal promoter, the next step was to more precisely locate the cis-acting elements involved in sugar responsiveness. Various 10-bp fragments containing EcoRI sites (GAATTC) were introduced into individual constructs to replace various regions within SRS in p3Luc.18, thus generating constructs p3Luc.28 through
p3Luc.36, as shown in Fig. 6A. These constructs were then tested for luciferase activity and the results are shown in Fig. 6B. All the linker substitutions had more or less effect on expression as compared with the wild type sequence. The GC box can be further divided into three GC-rich subdomains designated as GC1, GC2, and GC3 boxes. The GC2 and GC3 boxes each contain the identical 9-bp sequence CCGACGCGG. Mutations in the GC2 box (p3Luc.29) and GC3 box (p3Luc.30) resulted in 40 and 60% reduction of expression, respectively. Two mutations in the duplicated TATCCA element (p3Luc.33 and p3Luc.34) caused dramatic reduction in the level of glucose starvation-induced expression to 12 and 8% of the control (p3Luc.18), respectively. Mutations in the G box (p3Luc.31) resulted in a 80% reduction of expression. These results demonstrate that all of the sequences within SRS are necessary, and that the GC3 box, the G box, and the TATCCA element are the most important sequences for high level glucose starvation-induced expression.

**Nuclear Proteins Binding to the cis-Acting Elements in SRS**—Since the 52-bp fragment containing the TATCCA element enhanced glucose starvation-induced transcriptional activity (Fig. 4) and the TATCCA element is essential in conferring sugar-dependent regulation (Fig. 6), we examined whether nuclear proteins from rice suspension cells bind to the TATCCA element. DNA fragments encompassing various regions of SRS were synthesized and designated as F1 through F5 (Fig. 7A). These fragments were assayed for their ability to interact with nuclear protein extract from rice suspension cells grown in the

**FIG. 4**. A 52-bp fragment containing the TATCCA element enhances sugar-dependent transcription. Multiple copies of fragment −133 to −82 containing the duplicated TATCCA element was fused upstream of the 35S minimal promoter as described under “Experimental Procedures.” These constructs were transfected into rice protoplasts and luciferase activity was determined as described in the legend to Fig. 2.

**FIG. 5**. SRS converts the sugar-insensitive Act1 promoter into a sugar-sensitive promoter. SRS was inserted in one, two, or three tandem copies into the EcoRI site (−459) of the rice Act1 promoter as described under “Experimental Procedures.” These constructs were transfected into rice protoplasts and luciferase activity was determined as described in the legend to Fig. 2.
Presence or absence of sucrose. In the gel mobility shift assay using F2, which contains the TATCCA element, as the probe, two DNA-protein complexes (C1 and C2) were observed regardless of whether the nuclear extract was from cells grown in the presence of sucrose (1S) (Fig. 7B, lane 2) or in the absence of sucrose (2S) (Fig. 7B, lane 3). However, the band intensity between F2 and the nuclear extract was 5-fold higher for 2S cells than that for 1S cells. C1 and C2 were competed out by a 500-fold amount of F2 itself (Fig. 7B, lanes 6 and 11) and a 150-fold amount of F5 which contains F2 (Fig. 7B, lanes 9 and 14).

In the gel mobility shift assay using F3, which contains the G box and 5'-flanking sequence of the TATCCA element, one DNA-protein complex (C3) was also observed regardless of

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**Fig. 6. Linker-scan mutation analysis of SRS identified essential elements required for sugar-dependent regulation.** Plasmids carrying a series of linker-scan mutagenized SRS fused upstream of the CaMV35S minimal promoter were constructed by PCR as described under “Experimental Procedures.” A, nucleotide sequences of the wild type SRS and the linker-scan mutated SRS. Sequences different from the wild type promoter are shown in lowercase. B, constructs in A were transfected into rice protoplasts and luciferase activity was determined as described in the legend to Fig. 2.
FIG. 7. Gel mobility shift assay identified nuclear protein factors binding to the TATCCA element in a sequence-specific and sugar-dependent manner. A, diagram showing the sequences and positions of synthetic oligonucleotides within SRS that were used for gel mobility shift assay. B-D, gel mobility shift assays. Nuclear proteins were prepared from rice suspension cells that had been grown in the presence (+) or absence (−) of sucrose (S) for 2 days. The labeled F2 (B), F3 (C), or TATA box (D) was used as a probe. Sequence of oligonucleotide containing the TATA box is GTTCTATATATGCCCCC (position of TATA box underlined). Lanes 1 and 4, no nuclear protein; lanes 2 and 3, no competitor DNA; lanes 5–8 and 10–13, 500-fold excess (w/w) of competing DNA; lanes 9 and 14, 150-fold excess (w/w) of competing DNA. Positions of the DNA-protein complexes, C1, C2, and C3, and the free probe are shown with arrows.
whether the nuclear extract was from +S cells (Fig. 7C, lane 2) or −S cells (Fig. 7C, lane 3). However, the band intensity between F3 and the nuclear extract was 4.5-fold higher for +S cells than that for −S cells. C3 was competed out by a 500-fold amount of F3 itself and F4 (Fig. 7C, lanes 7 and 8 and lanes 12 and 13) and a 150-fold amount of F5 (Fig. 7C, lanes 9 and 14). Because F3 contains a 7-bp sequence, TTATTG (positions −126 to −120), which is also present in F4 and F5 (positions −99 to −93), we believe that C3 was formed between this 7-bp sequence with a nuclear protein. F2 competed slightly for C3 probably because it contains a 4-bp sequence ATTTG which overlaps part of the 7-bp sequence (Fig. 7C, lanes 6 and 11).

An additional gel shift assay using a DNA fragment containing the TATA box of αAmy3 as a probe, two to three DNA-protein complexes were observed regardless of whether the nuclear extract was from +S cells (Fig. 7D, lane 2) or −S cells (Fig. 7D, lane 3). The band intensity of the complexes was similar between the two nuclear extracts. These complexes were not competed out by a 500-fold amount of F2 (Fig. 7D, lanes 5 and 7), but were competed out by the TATA box itself (Fig. 7D, lanes 6 and 8). This result demonstrates that the apparent differences in the levels of the SRS DNA binding factors shown in Fig. 7, B and C, are due to changes in the functional amounts of the factors and not due to extraction and/or solubilization of nuclear factors. The above results also demonstrate that nuclear proteins from rice suspension cells bind to the TATCCA element and its flanking sequences in a sequence-specific and sugar-dependent manner.

**DISCUSSION**

The GC Box, G Box, and TATCCA Element Act Synergistically in Regulating Sugar-dependent Expression of a Minimal Promoter—Linker-scan mutation analysis identified the GC3 box, G box, and TATCCA element within SRS as the most essential sequences to confer high-level glucose starvation-induced expression. Fragment −186 to −122, which contains the GC box and G box (p3Luc.19) only, dramatically reduced the glucose starvation-induced expression of the 35S minimal promoter (Fig. 3). Such reduction was probably not simply due to an alteration in the distance between these sequences and the TATA box, because mutation of the TATCCA element within SRS (p3Luc.33 and p3Luc.34) without alteration in the distance still resulted in significant reduction of glucose starvation-induced expression (Fig. 6). Apparently, the TATCCA element is an indispensable element for high-level expression. The 52-bp fragment (−133 to −82), which contains the duplicated TATCCA element only (p3Luc.40), shows no induction in Fig. 3, but an 1.8-fold induction in Fig. 4. The discrepancy was probably due to technical difficulty and variation in preparation and electroporation of rice protoplasts from one experiment to another, but the variation always remained below a factor of two in three repeated experiments for each construct. Therefore, the luciferase activity from p3Luc.40 is not significantly different between cells grown with and without glucose and are close to background. Duplication of the 52-bp fragment (p3Luc.41) recovered the glucose starvation-induced expression, indicating that stable interaction between trans-acting factor(s) and the TATCCA element requires neighboring sequence around −133 and/or −82. Mutations in either the downstream or upstream copy of the TATCCA element (p3Luc.41 m1 and p3Luc.41 m2) reduced the starvation-induced expression which suggests both the sequence of TATCCA element and the distance relative to TATA box are essential for expression.

The extent of sugar repression from either construct p3Luc.19x2 (7-fold repression) or construct p3Luc.41 (4-fold repression) is significant between cells grown with and without glucose, however, the fold repression between the two constructs are not significantly different. Sequence containing either the GC box plus G box or the TATCCA element each can confer sugar-dependent regulation on the 35S minimal promoter. However, presence of both sequences contribute to high level glucose starvation-induced expression, suggesting that these sequences act synergistically and their association was termed a sugar response complex.

The 52-bp Fragment Containing the TATCCA Element Enhances Sugar-dependent Transcription—It is interesting to note that the absolute level of expression from more than four copies of the 52-bp fragment containing the TATCCA element far exceeded that from SRS regardless of the glucose concentration (Fig. 4). Particularly, the 52-bp fragment can substitute for the GC box and G box for conferring high level glucose starvation-induced expression. Multiple copies of the 52-bp fragment enhanced transcription regardless of the glucose concentration, which suggests that transcription factors binding to the cis-acting element (possibly the TATCCA element) is constantly present in the nucleus. More copies of the 52-bp fragments may recruit more transcription factors to the promoter region. The higher level of expression induced by glucose starvation could be due to an increase in the abundance of transcription factors or in the affinity for binding between the cis-acting element and the transcription factors under glucose starvation. On the other hand, the fold induction of luciferase activity from SRS under glucose starvation was higher than that from multiple copies of the 52-bp fragment (Fig. 4), suggesting that an additional sequence(s) is required for the sugar repression of gene expression. The additional sequence(s) must be present between −186 and −133.

The TATCCA Element Enhances GA- and Sugar-dependent Promoter Activities—Examination of promoter sequences of nine rice, nine barley, and five wheat α-amylase genes that are available in GenBank reveals that almost all except four of these genes have TATCCA variants, and that they all contain a TATCCA element at positions approximately 100 to 150 bp upstream of transcription start sites. This observation suggests that the TATCCA element may play a role in the regulation of α-amylase gene expression. Mutations of the TATCCA element in the promoters of both barley high-PI α-amylase gene Amy pHV19 (28) and low-PI α-amylase gene Amy32b (29) were found to lower expression to about 20% of maxima but maintained GA responsiveness. In our study, mutation of the duplicated TATCCA element (p3Luc.33 and p3Luc.34) also reduced the Amy3 promoter activity to 12 and 8%, respectively, of the wild type sequence (p3Luc.18) but maintained sugar responsiveness (Fig. 6). These results suggest that the TATCCA element enhances transcription in concert with the hormone- or sugar-regulated interactions on other sequences to promote the transcription of α-amylase promoters in an environmental signal- and tissue-dependent manner. The function of the TATCCA element, possibly with the involvement of its flanking sequences, seems to operate somewhat differently between the GA- and sugar-dependent regulatory systems, i.e. GA control of expression exerted by the promoter fragments containing the GA response element and TATCCA element from barley Amy pHV19 or rice OSAmy-c is orientation-dependent (28, 33), whereas the sugar control of expression exerted by the promoter fragment containing SRS from aAmy3 is orientation-independent (Fig. 3).

In addition to the TATCCA element, the G box was also identified as an essential component of the sugar response complex. The G-box is present in the 5′-upstream regions of plant genes exhibiting regulation by a variety of environmental signals and physiological cues (51). Taken together, the above
studies suggest that the regulation of α-amylase gene expression by environmental factors, i.e., osmotic stress (8) and physiological cues (gibberellin or sugar signal), may share some common regulatory machinery. These findings provide new insight into the mechanisms involved in the regulation of α-amylase gene expression in cereals.

**SRS Converts a Sugar-insensitive Promoter into a Sugar-sensitive Promoter.—**The expression of the Act1 promoter-GUS gene (data not shown) or Act1 promoter-Luc (Fig. 5, pActLuc) in rice protoplasts is normally not significantly affected by glucose. Insertion of SRS in the −459 position of the Act1 promoter alters the mode of regulation of this promoter by glucose. Previously, the Act1 promoter deletion to nucleotide −459 has been shown to still display high activity in rice protoplasts (53), suggesting that conversion of the promoter activity in response to glucose is not simply due to disruption of the promoter sequence by SRS. It is interesting to note that the absolute level of glucose starvation-induced expression of the Act1 promoter containing SRS was dramatically higher than that of the 35S minimal promoter fused to SRS (compare Fig. 5 with Fig. 3), suggesting that SRS may enhance the transcription of a promoter by a much higher magnitude if the promoter has full function.

The enhancement of transcription exerted by multiple copies of SRS under glucose starvation could be due to stable and/or multiple interactions between trans-acting factors and the sugar response element(s) within SRS. An activator which enhances transcription in proportion to the copy number of SRS is likely to be involved in the glucose starvation-induced expression of the αAmy3 promoter. This notion is also supported by the linker-scan mutation study in which the expression of the 35S minimal promoter fused to various mutated SRS varied significantly in low concentration glucose, indicating that the activator binding sites are affected by various mutations. Taken together, all of these observations suggest that glucose repression involves repression of a functional activator(s) which otherwise interacts with SRS and activates transcription.

**Nuclear Protein Factors Binding to the TATCCA Element and Its Flanking Sequences in a Sequence-specific and Sugar-dependent Manner.—**An important step toward understanding the molecular mechanism of sugar-dependent regulation is the identification of trans-acting regulatory proteins. By gel mobility shift assay, nuclear proteins from rice suspension cells were found to specifically bind to the TATCCA element and its flanking sequences (Fig. 7, B and C). The protein factors were present in the nuclei of +S or −S cells, which suggests that the protein factors are constantly present in the nuclei. Higher amounts of complex formations between the nuclear proteins from the −S cells and the TATCCA element could be due to the existence of higher amounts of transcription factors in the −S cells, or the higher binding affinity of transcription factors with the TATCCA element under sucrose starvation. Higher amounts of complex formations between the nuclear protein(s) from the +S cells and the flanking sequences of the TATCCA element can be similarly explained. However, protein factors bind to the TATCCA element and its flanking sequences may serve opposite functions, activator and repressor, respectively. Although the data shown in Figs. 5 and 6 suggest that activators may be involved in the sugar starvation-induced αAmy3 expression, the possibility that the repressor is also involved in the sugar repression of αAmy3 expression cannot be ruled out. This possibility is supported by an observation that the protein synthesis inhibition cycloheximide enhanced transcription of the αAmy3 promoter (data not shown), probably through an inhibition of repressor synthesis. As judged from the involvement of multiple cis-acting elements in the sugar response and the complexity of the protein–protein interaction patterns, the transcription factors may be post-translationally modified, recruit additional proteins, or exhibit specificity in their interaction with other transcription factors.

**Conclusion.—**In the last decade, molecular mechanism of GA regulation of α-amylase gene expression have been extensively studied and functional analyses have identified several promoter sequences important for the GA response. Despite the fact that sugar also serves as an essential signal in controlling the expression of α-amylase genes in cultured rice suspension cells (17), in germinating rice seed (8, 16), and in germinating barley embryo (54, 55), to date the sugar response sequence has been studied only for the rice αAmy3. In addition, although carbohydrate depletion induces expression of a variety of genes involved in photosynthesis, reserve mobilization, and export processes (56), the cis-acting sugar response elements in the promoters of these genes have not been precisely defined either. A 20-bp sequence (at position −90 to −70 upstream of the transcription start site) in a Drosophila α-amylase gene promoter was shown to be necessary for full activity of this promoter in transformed larvae (25). However, this 20-bp sequence does not contain a GC box or G box-like sequence or TATCCA element. Conserved cis-acting elements have not yet been found among promoters of sugar repressible genes in plants, Drosophila, and yeasts. Our studies have initiated an important question as to whether the mechanisms through which sugar regulates gene expression are conserved or diverged throughout the evolution of different kingdoms.

In summary, our studies present identification of functional sugar response elements and the existence of interacting trans-acting factors which regulate sugar repressible genes in plants. These results should lay the foundation for the eventual elucidation of the signal transduction pathway leading to sugar feedback regulation of gene expression in plants. In addition, identification of the TATCCA-binding protein would also facilitate study of the complex regulatory network in which α-amylase genes respond to sugars and gibberellins for conversion of stored starch into nutrients in germinating cereal grains (8).

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Sugar Regulation of α-Amylase Gene

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