Rice \( \alpha \)-Amylase Transcriptional Enhancers Direct Multiple Mode Regulation of Promoters in Transgenic Rice*

Received for publication, October 9, 2001, and in revised form, February 1, 2002
Published, JBC Papers in Press, February 6, 2002, DOI 10.1074/jbc.M109722200

Peng-Wen Chen‡, Chung-An Lu‡, Tien-Shin Yu‡, Tung-Hi Tseng§, Chang-Sheng Wang§, and Su-May Yu‡¶

From the §Institute of Molecular Biology, Academia Sinica, Nankang, Taipei, Taiwan 115, Republic of China and the ¶Taiwan Agricultural Research Institute, Wu-Fong, Taichung, Taiwan 413, Republic of China

Expression of \( \alpha \)-amylase genes in cereals is induced by both gibberellin (GA) and sugar starvation. In a transient expression assay, a 105-bp sugar response sequence (SRS) in the promoter of a sugar starvation highly inducible rice \( \alpha \)-amylase gene, \( \alpha \)Amy3, was shown previously to confer sugar response and to enhance the activity of the rice Act1 promoter in rice protoplasts. A 230-bp SRS-like sequence was also found in the promoter of another sugar starvation highly inducible rice \( \alpha \)-amylase gene, \( \alpha \)Amy8. The \( \alpha \)Amy8 SRS contains a GA response sequence and was designated as \( \alpha \)Amy8 SRS/GARS. In the present study, a transgenic approach was employed to characterize the function of the \( \alpha \)-amylase gene SRSs in rice. We found that the \( \alpha \)Amy3 SRS significantly enhances the endogenous expression pattern of the Act1 promoter in various rice tissues throughout their developmental stages. By contrast, the \( \alpha \)Amy8 SRS/GARS significantly enhances Act1 promoter activity only in embryos and endosperms of germinating rice seeds. A minimal promoter fused to the \( \alpha \)Amy8 SRS/GARS is specifically active in rice embryo and endosperm and is subject to sugar repression and GA induction in rice embryos. This sugar repression was found to override GA induction of \( \alpha \)Amy8 SRS/GARS activity. Our study demonstrates that the \( \alpha \)-amylase transcriptional enhancers contain cis-acting elements capable of enhancing endogenous expression patterns or activating sugar-sensitive, hormone-responsive, tissue-specific, and developmental stage-dependent expression of promoters in transgenic rice. These enhancers may facilitate the design of highly active and tightly regulated composite promoters for monocot transformation and gene expression. Our study also reveals the existence of cross-talk between the sugar and GA signaling pathways in cereals and provides a system for analyzing the underlying molecular mechanisms involved.

\( \alpha \)-Amylases were originally noted for their expression as regulated by GA\(^1\) and their importance in starch utilization in germinating cereal grains (1). During germination of cereal grains, the embryo synthesizes GA that diffuses to the aleurone cells and acts as a signal to activate the synthesis and secretion of \( \alpha \)-amylases and other hydrolyases. These enzymes digest the starch stored in the endosperm and provide sugars for the growth of young seedlings. We now know that expression of \( \alpha \)-amylase genes is activated by sugar deprivation and repressed by sugar provision in cultured rice suspension cells (2, 3) and in the embryos of germinating rice (4) and barley (5) seeds. This sugar regulation of \( \alpha \)-amylase gene expression has recently become a model system for studying the molecular mechanisms that mediate sugar repression in plants (6, 7).

Sugar repression of \( \alpha \)-amylase gene expression involves control of both transcription rate and mRNA stability (8–10). The \( \alpha \)Amy3 SRS was shown to confer sugar responsiveness to a cauliflower mosaic virus 35S RNA (CaMV35S) minimal promoter in an orientation-independent manner (11). The \( \alpha \)Amy3 SRS contains three essential motifs: the GC box, the G box, and the TATCCA element (Fig. 1a) for a high level of sugar starvation-induced gene expression in rice protoplasts (11). All of the \( \alpha \)-amylase genes isolated from rice, barley, and wheat contain a TATCCA element or its variants at the proximity of −90–150 bp, upstream of transcription start sites (6). Mutations of the TATCCA element in the promoters of two barley \( \alpha \)-amylase genes, Amy-pHV19 (12) and Amy32b (13), lowered expression to about 20% of the wild-type sequence but maintained GA responsiveness in the barley aleurone. The TATCCA element is duplicated in the \( \alpha \)Amy3 promoter, and mutation of each of the duplicated TATCCA elements also reduced the \( \alpha \)Amy3 promoter activity to 12% and 8%, respectively, of the wild-type sequence but maintained sugar starvation inducibility in the rice protoplasts (11). The TATCCA element significantly enhanced transcription of the CaMV35S minimal promoter in rice protoplasts in a dose- and glucose-dependent manner, suggesting that the TATCCA element serves as a transcriptional enhancer (11).

One of the most important considerations in developing a plant transformation procedure is the availability of a promoter that provides a reliable high level expression of introduced genes in target cells or tissues. Monocot plants, particularly the cereal species, comprise an economically important group of plant species that could benefit from the introduction and expression of foreign genes that control agronomically important traits and overproduction of biomolecules. The rice Act1 promoter is one of the most frequently used highly active and constitutive promoters (14) in the establishment of transformation procedures and expression of foreign genes in rice and other monocots. Another constitutive promoter that is also commonly used for transformation of monocots is the maize Ubi promoter (15). In general, the maize Ubi promoter has slightly

---

* This work was supported by a grant from Academia Sinica, Grant NSC-90-2311-B-001-008 from the National Science Council, and a grant from the Biomedical Research Foundation of the Republic of China. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed. Tel.: 886-2-2788-2695, Fax: 886-2-2788-2695 or 886-2-2782-6085, E-mail: sumay@ccvax.sinica.edu.tw.

¶ The abbreviations used are: GA, gibberellin; GARE, GA response element; GARS, GA response sequence; SRS, sugar response sequence; 2,4-D, 2,4-dichlorophenoxyacetic acid.
higher activity than the rice Act1 promoter in various monocot species (16–18). However, we found that the maize Ubi and rice Act1 promoters frequently do not lead to a high level of foreign gene expression in transgenic rice.

Various strategies can be employed to increase the activity of constitutive promoters in transformed cereals (16, 18). One strategy is to add an enhancer element that increases the transcription of a promoter. In plants, extra copies of enhancer elements can enhance the activity of a homologous promoter, e.g., the anaerobic responsive element to the maize Adh1 promoter (19). Multiple copies of enhancer elements have also been employed to enhance the activity of a heterologous promoter, e.g., the octopine synthase enhancer from Agrobacterium tumefaciens to the maize Adh1 promoter (20). Previously, we found that insertion of three tandem copies of the rice αAmy3 SRS in the Act1 promoter significantly enhances the promoter activity in rice protoplasts in a dose-dependent manner (11).

Unlike the αAmy3 SRS, SRSs in promoters of other sugar starvation-inducible genes have not been extensively characterized. Previously, we showed that the GC box and TATCCA element are both present in the promoter of a starvation-inducible gene (11). In the present study, we demonstrate that the GC box and TATCCA element are both present in the promoter of αAmy3, the αAmy8 promoter, and tissue-specific expression of promoters in transgenic rice.

**EXPERIMENTAL PROCEDURES**

**Plant Material**—The rice variety used in this study was Oryza sativa L. cv. Taizung 67. Immature seeds were dehulled, sterilized with 3% NaOCl for 30 min, washed extensively with sterile water, and placed on N6D agar medium (41) for callus induction. After 1 month, calli derived from scutellum were subcultured in fresh N6D medium for transformation.

**Plasmid Construction**—For construction of the plasmid containing the Act1–Luc chimeric gene, the EcoRI site in the multiple cloning sites of pBluescript SKII+ (Stratagene) was first removed by digestion with EcoRI and then blunt-ended and re-ligated. The rice Act1 5′ region (including 1.4 kb 5′-flanking sequence, 79-bp 5′ noncoding exon, 447-bp 5′ intron, and 25-bp first coding exon) was excised from pDM302 (42) with HindIII and subcloned into the same site in pBluescript, which lacked the EcoRI site to generate pAct. The αAmy3 SRS (−186 to −82 relative to the transcription start site of αAmy3) was PCR-amplified using oligonucleotides 5′-CCCGAATTCATCCCGTCGCCTTGGAGA-3′ and 5′-CCCGAATTCGAGGACGTTTTGAAACTGAG-3′ as the DNA template. The DNA fragment containing the SRS was digested with EcoRI and inserted into the EcoRI site underlined as the 5′ primer and 5′-CGCAATTCCAGAGACGTTTTGAAACTGAG (EcoRI site underlined) as the 3′ primer and p3G-123II (11), which contains the 1.7-kb 5′ region of αAmy3, as the DNA fragment.

**Transformation of Rice**—Plasmids pAct-LN and pAct-SRS-LN were linearized with PstI and inserted into the Smal site of pAct and pAct-SRS to generate pAct-LN and pAct-SRS-LN. pB-ACT-3SRS-LN was linearized with PstI and inserted into the binary vector pSY112 (39), which contains the 3SS promoter Hph coding region tumor morphology large gene (Tml) terminator fusion gene, thereby generating pAct-LN and pAct-SRS-LN.

**Transformation of Rice**—Plasmids pAct-LN and pAct-SRS-LN were introduced into A. tumefaciens strain EHA101 (44) with an electroporator (BTX, San Diego, CA) according to the manufacturer’s instructions. Calli induced from immature rice seeds were co-cultured with A. tumefaciens, and putative transgenic plants were regenerated from calli according to the methods described by Hiei et al. (36) and Toki (41).

**Suspension Cell Culture**—Transformed calli were propagated as described previously (2). Established suspension cells were subcultured as described previously (11).

**Luciferase Activity Assay**—Total proteins were extracted from cultured suspension cells or plant tissues with a CCLR buffer (100 mM KH2PO4, pH 7.8, 1 mM EDTA, 10% glycerol, 1% Triton X-100, 7 mM β-mercaptoethanol), and the protein concentration was determined with a Coomassie protein assay reagent (Pierce). Luciferase activity assay was performed as described previously (11).

**Nucleotide sequences of the αAmy3 SRS and αAmy8 SRS/GARS**. a, the sequences are numbered relative to the transcription start sites (+1) of the αAmy3 and αAmy8 promoters (11). b, nucleotide sequence comparison of the GC box present in αAmy3 SRS and αAmy8 SRS/GARS.
**RESULTS**

The **Amy3 SRS Enhances Act1 Promoter Activity in Transformed Rice Suspension Cells**—Previously, we showed in a protoplast transient expression assay that insertion of three copies of the **Amy3** SRS at position 459 bp upstream of the transcription start site of an 846-bp Act1 promoter significantly enhanced Act1 promoter activity (11). To determine whether similar results could be produced in stably transformed rice cells, the firefly luciferase gene (Luc) was fused downstream of the Act1 promoter or the Act1 promoter containing three tandem copies of **Amy3** SRS, generating Act1-Luc and Act1-3SRS-Luc, and the resulted chimeric genes (Fig. 2) were introduced into the rice genome via Agrobacterium-mediated transformation. Several transformed cell lines were obtained, and four lines for each construct were randomly selected for further study. The transformed calli were cultured as suspension cells. These cells were then cultured in medium with or without sucrose for 2 days prior to luciferase assay. The luciferase activity conferred by the wild-type Act1 promoter was very low in sucrose-starved cells and was 6- to 8-fold less than in sucrose-provided cells (Fig. 3). By contrast, the luciferase activity conferred by the Act1-3SRS promoter increased dramatically in sucrose-starved cells to 2-5.5-fold of that in sucrose-provided cells (Fig. 3). Notably, the Act1-3SRS promoter conferred significantly higher luciferase activity regardless of whether cells were cultured with or without sucrose. These results suggest that in stably transformed rice suspension cells, expression of luciferase is significantly enhanced by integration of the **Amy3** SRS into the Act1 promoter. Additionally, the **Amy3** SRS converts the sugar-inducible Act1 promoter into a sugar starvation-inducible promoter.

The **Amy3 SRS Generally Enhances Act1 Promoter Activity in Transgenic Rice**—To determine whether integration of the **Amy3** SRS into the Act1 promoter enhances expression of luciferase in transgenic rice plants, transformed calli carrying Act1-Luc and Act1-3SRS-Luc chimeric genes were regenerated and then self-fertilized for three generations to obtain T3 homozygous seeds. The homozygosity of transgenic seeds was determined by the germinating 25 transgenic seeds in water containing 50 μg/ml hygromycin for 7 days and then calculating the ratio between the number of growing and non-growing seedlings. Theoretically, seeds of a transgenic line homozygous for the transgene should all germinate and grow under these conditions. T3 homozygous seeds of four transgenic lines carrying Act1-Luc and eight transgenic lines carrying Act1-3SRS-Luc were germinated and grown for 8 days. Leaves were collected from seedlings and assayed for luciferase activity. The luciferase activity was not significantly different in the leaves of all transgenic lines carrying the same construct. The average luciferase activity conferred by the Act1-3SRS promoter in different transformants was similar: 2.5- to 3-fold higher than that conferred by the Act1 promoter (Fig. 4). These results indicate that the **Amy3** SRS generally enhances Act1 promoter activity in transgenic rice.

The **Amy3 SRS Enhances Developmentally Regulated Act1 Promoter Activity in Various Tissues of Transgenic Rice**—To determine whether the Act1-3SRS chimeric promoter enhances expression of luciferase in different tissues of germinating seeds and seedlings, T3 homozygous seeds of transgenic lines Act6-9-1 and Act(3SRS) 5-15-1 were germinated and grown for 2 weeks. Various tissues were collected and assayed for luciferase activity. The Act1-3SRS promoter conferred higher luciferase activity than the Act1 promoter in various tissues of germinating seeds and seedlings (Fig. 5). The difference in enhancement of luciferase activity was most dramatic in roots (5- to 56-fold) and next in shoots (4- to 11-fold) and embryos (3- to 18-fold). The luciferase activity in various organs conferred by the Act1 and Act1-3SRS promoters peaked within 7-8 days after germination and declined thereafter.

To determine the luciferase expression pattern under control of the Act1-3SRS promoter in seedlings and older plants, T3 homozygous seeds of transgenic lines Act 6-9-1 and Act(3SRS) 5-15-1 were germinated and grown for 8 weeks. Various tissues were collected and assayed for luciferase activity. The Act1-3SRS promoter conferred significantly higher luciferase activity than the Act1 promoter in various tissues of transgenic rice plants (Fig. 6a). The difference in enhancement of luciferase activity was highest in root (7- to 38-fold), next highest in leaf (5- to 20-fold), and lowest in sheath (4- to 7-fold). The total luciferase activity in the leaf, sheath, and root of transgenic seedlings or plants as described in Fig. 6a was calculated and compared (Fig. 6b). The data shown in Figs. 5 and 6 demonstrate that the luciferase activity in transgenic rice, conferred by both the Act1 and Act1-3SRS promoters, fluctuates in a developmental stage-dependent manner. The luciferase activity in various tissues reached its first peak within 1 week after germination, reached its lowest level at week 2 or 3, and rose again at week 4.

---

**Fig. 2.** Expression cassettes for rice transformation. Plasmid pAct-LN contains the wild-type Act1 promoter. pAct-3SRS-LN contains three tandem repeats of the **Amy3** SRS, and pAct-8SRS/GARS-LN contains three tandem repeats of the **Amy3** SRS/GARS inserted in the EcoRI site (459 bp upstream of the transcription site) of the Act1 promoter. p8SRS/GARS-35S-LN contains one copy of the **Amy3** SRS/GARS fused upstream of the CaMV35S minimal promoter. Luc was transcriptionally fused downstream of the above promoters and upstream of the nopaline synthase gene terminator (Nos 3).

**Fig. 3.** The **Amy3** SRS enhances Act1 promoter activity in transformed rice suspension cells. Transformed rice suspension cells carrying Act1-Luc or the Act1-3SRS-Luc gene were cultured in the presence of sucrose (+ sucrose, open bar) or absence of sucrose (− sucrose, filled bar) for 2 days. Cells were collected, and luciferase activity was determined by four independently transformed rice cell lines for each construct.
To determine whether growth conditions affect the efficacy of the \( \alpha / H9251 \) Amy3 SRS in enhancing \( \text{Act1} \) promoter activity, T3 homozygous seeds of transgenic lines Act 6-9-1 and Act(3SRS) 5-15-1 were allowed to germinate and grow in the dark or in a 12-h light/12-h dark cycle for 12 days. Leaves of transgenic rice seedlings were collected and assayed for luciferase activity. The luciferase activity conferred by the \( \text{Act1}-3\text{SRS} \) promoter was significantly higher than that conferred by the \( \text{Act1} \) promoter, regardless of whether seedlings were grown in the dark or under the light/dark cycle condition (Fig. 7). The luciferase expression in leaves of seedlings grown under light/dark cycles was also higher than in leaves grown in the dark within the first week after germination regardless of whether or not the \( \text{Act1} \) promoter contained the \( \alpha / H9251 \) Amy3 SRS.

Enhancement of \( \text{Act1} \) Promoter Activity by the \( \alpha / H9251 \) Amy3 SRS Is Tissue-independent, Whereas That of the \( \alpha / H9251 \) Amy8 SRS/GARS Is Tissue-dependent—The rice \( \alpha / H9251 \) amylases are encoded by a multigene family (21). In the present study, we first compared the expression patterns of the \( \alpha / H9251 \) -amylase genes in various rice tissues to understand how they are regulated. As shown in Fig. 8, the \( \alpha / H9251 \) -amylase genes are differentially expressed in rice suspension cells and germinated seeds. Although expression of all the \( \alpha / H9251 \) -amylase genes was enhanced by sugar starvation, the magnitude of enhancement varied from gene to gene (Fig. 8, compare lane 1 with lane 2). All \( \alpha / H9251 \) -amylase genes were barely expressed in shoots and roots, but most of them were expressed in endosperms and embryos of germinated rice seeds. Both \( \alpha / H9251 \) Amy3 and \( \alpha / H9251 \) Amy8 were highly expressed in sucrose-starved cells; however, only \( \alpha / H9251 \) Amy8 was highly expressed in endosperms. These results indicate that \( \alpha / H9251 \) Amy3 and \( \alpha / H9251 \) Amy8 are coordinately regulated by sugar in rice suspension cells but differentially regulated, presumably by GA, in germinating seeds. This expression of \( \alpha / H9251 \) -amylase genes is regulated by GA at the transcription level in endosperm (22, 23) and by sugars in rice suspension cells (8, 9). The coordinated and differential regulation could be due to the presence of conserved or distinct regulatory elements in the \( \alpha / H9251 \) Amy3 and \( \alpha / H9251 \) Amy8 promoters. We therefore compared the cis-acting elements in the two promoters.

The rice \( \alpha / H9251 \) Amy8 promoter was shown previously to drive sucrose starvation-induced expression of a reporter gene in rice suspension cells (8). Sequence analysis revealed that the \( \alpha / H9251 \) Amy8 promoter contains a 31-bp GC box at positions −266 to −236 and a TATCCA element at positions −131 to −126 up-
stream of the transcription site (Fig. 1a). The nucleotide sequences of the /H9251 Amy3 and /H9251 Amy8 GC boxes are highly homologous (Fig. 1b). The 230-bp /H9251 Amy8 SRS/GARS, which encompasses positions /H11002 318 to /H11002 89 of the /H9251 Amy8 promoter, contains the GC box and TATCCA element (Fig. 1a). Between the GC box and the TATCCA element, two additional sequences homologous to the c-Myb-binding site (24) and GARE (25) are present in the /H9251 Amy8 SRS/GARS but are absent in the /H9251 Amy3 SRS. To test whether the /H9251 Amy8 SRS/GARS may also serve as an enhancer, this sequence was also inserted at position /H11002 459 of the Act1 promoter and tested for its effect on Act1 promoter activity in transgenic rice.

The patterns of luciferase expression conferred by the Act1-3SRS and Act1-SRS-Luc chimeric promoters (Fig. 2) in various tissues of transgenic rice seedlings were compared. T3 homozygous seeds of transgenic lines Act 6-9-1, Act(SRS) 5-15-1, and Act(8SRS) 5 were germinated and grown for 8 days. Every 2 days, leaves were collected from five seedlings of each transgenic line and assayed for luciferase activity. Error bars indicate the standard deviation of luciferase activity in the five seedlings.

FIG. 6. The /Amy3 SRS enhances developmentally regulated Act1 promoter activity in various tissues of transgenic rice. T3 homozygous seeds of transgenic lines Act 6-9-1 and Act(SRS) 5-15-1 carrying the Act1-Luc gene (open column) and Act1-SRS-Luc gene (filled column), respectively, were germinated and grown in a greenhouse for 8 weeks (a). After each week, leaves, sheaths, and roots were collected from five plants and assayed for luciferase activity. Error bars indicate the standard deviation of luciferase activity in five plants. Average luciferase activity in leaf (hatched box), sheath (open box), and root (filled box) of T3 transgenic rice lines Act 6-9-1 and Act(SRS) 5-15-1 determined each week as shown in panel a is summed and presented as stacked columns (b).

FIG. 7. The efficacy of /Amy3 SRS in enhancement of Act1 promoter activity is not affected by growth conditions of transgenic rice. T3 homozygous seeds of transgenic lines Act 6-9-1 and Act(SRS) 5-15-1 carrying the Act1-Luc gene and Act1-3SRS-Luc gene, respectively, were germinated and grown in the dark (filled column) or in 12-h light/12-h dark cycles (open column) for 12 days. Every 2 days, leaves were collected from five seedlings of each transgenic line and assayed for luciferase activity. Error bars indicate the standard deviation of luciferase activity in the five seedlings.

The /Amy3 SRS/GARS Confers Endosperm- and Embryo-specific Activity to a Minimal Promoter—Since the /Amy3 SRS/GARS enhanced Act1 promoter activity significantly in embryos, shoots, and roots, whereas the /Amy8 SRS/GARS enhanced Act1 promoter activity mainly in endosperms and embryos of germinated seeds and seedlings. The /Amy8 SRS/GARS Confers Endosperm- and Embryo-specific Activity to a Minimal Promoter—Since the /Amy8 SRS/GARS enhanced Act1 promoter activity mainly in endosperms and embryos of germinated seeds and seedlings, we next wished to determine whether the /Amy8 SRS/GARS itself confers tissue-specific activity to a minimal promoter. The /Amy8 SRS/GARS
was fused upstream of the CaMV35S minimal promoter–Luc fusion gene (Fig. 2), which was then introduced into the rice genome. Several transgenic rice lines were obtained, and the T2 seeds of one randomly selected line, T4-12, were germinated for 6 days. Various tissues of germinated seeds were collected, and luciferase activity was assayed. As shown in Fig. 10, when compared with the activity in roots, the luciferase activity in endosperms and embryos was 74- and 20-fold, respectively. The activity in shoots was similar to that in roots. These findings show that the \( \alpha \mathrm{Amy8} \) SRS/GARS confers endosperm- and embryo-specific activity to a minimal promoter in germinated transgenic rice seeds.

The \( \alpha \mathrm{Amy8} \) SRS/GARS Confers Sugar and GA Responsiveness to a Minimal Promoter—\( \alpha \mathrm{Amy8} \) was expressed in sucrose-starved cells and endosperms, suggesting that the \( \alpha \mathrm{Amy8} \) promoter likely contains regulatory sequences responsible for sugar and GA responses. We decided to determine whether the \( \alpha \mathrm{Amy8} \) SRS/GARS confers the sugar and GA responsiveness to a minimal promoter. Embryos collected from rice seeds pretreated with 2,4-D have been shown to respond to exogenously applied sugar (26). Therefore, the same batch of T2 seeds from transgenic line T4-12 used in the experiment described in Fig. 10 was pretreated with 2,4-D for 8 days. Embryos were collected and divided into four groups. Each group of embryos was incubated with or without sucrose plus or minus GA for 2 days, and luciferase activity was assayed. As shown in Fig. 11a, in the presence of sucrose, luciferase activity in embryos was relatively low regardless of whether GA was present or not. In the absence of both sucrose and GA, luciferase activity in embryos increased significantly by 5.5-fold. The addition of GA in the absence of sucrose enhanced luciferase activity by 8.2-fold.

Embryos of T1 seeds from transgenic line T4-12 were removed in order to cut off the source of GA. The endosperms
were then divided into four groups. Each group of endosperms was incubated with or without sucrose plus or minus GA for 2 days, and luciferase activity was assayed. As shown in Fig. 11b, in the absence of GA, the luciferase activity in endosperms was low regardless of whether sucrose was present or not. In the presence of both GA and sucrose, the luciferase activity in endosperms increased by 4-fold, and the removal of sucrose did not alter the luciferase activity. These results demonstrate that the αAmy8 SRS/GARS confers GA responsiveness to a minimal promoter in both rice embryos and endosperms.

DISCUSSION

The Act1–3SRS Promoter Is a Strong Promoter for Foreign Gene Expression in Transgenic Rice—The availability of a promoter that provides reliable high level expression of introduced genes in target cells is an important issue in plant transformation. The maize Adh1 promoter, developed as an important promoter for monocot transformation, has low activity in monocots. A combination of six copies of the anaerobic responsive element enhancer and four copies of the octopine synthase enhancer with the maize Adh1 promoter, designated as Emu, significantly enhances the Adh1 promoter activity in protoplasts of maize, wheat, and rice in transient assays (27). The rice Act1 and maize Ubi promoters are now the most frequently used monocot promoters for monocot transformation. A comparison of promoter strength indicates that these monocot promoters exert differential strength in different monocot cells. For example, in barley suspension cells, Emu activity is 240% of the rice Act1 promoter and 130% of the maize Ubi promoter (17). However, in rice and maize suspension cells, Emu activity is only about 60% of the rice Act1 promoter and 40–50% of the maize Ubi promoter (17, 18).

In one of our previous studies involving rice protoplast transient expression assay, three copies of the αAmy3 SRS enhanced Act1 promoter activity 20-fold in glucose-starved rice protoplasts (11). In the present study, in stably transformed rice suspension cells, the αAmy3 SRS enhanced Act1 promoter

![Fig. 10.](image-url) The αAmy8 SRS/GARS confers endosperm- and embryo-specific activity to a minimal promoter in transgenic rice seedlings. T2 seeds of transgenic line T4-12 carrying the 8SRS/GARS-CaMV35S-Luc chimeric gene (Fig. 2) were germinated in water for 6 days. Endosperms, embryos, shoots, and roots of five germinated seeds were collected and assayed for luciferase activity. The value of luciferase activity in roots was assigned as 1X, and the other values relative to this value were then calculated. The experiment was repeated three times, and error bars indicate the standard deviation of luciferase activity.

![Fig. 11.](image-url) The αAmy8 SRS/GARS confers sugar and GA responsiveness to a minimal promoter in embryos of germinated seeds. T2 seeds of transgenic line T4-12 were incubated in liquid Murashige-Skoog medium containing 2 µg/ml 2,4-D for 8 days (a). Twenty embryos were dissected from the seeds, collected, and divided into four groups with five embryos in each group. Each group of embryos was incubated in the presence or absence of 100 mM sucrose with or without 0.1 mM GA for 2 days, and luciferase activity was assayed. Embryos were removed from 20 dry T2 seeds of transgenic line T4-12, and the endosperms were collected (b). Twenty endosperms were incubated in the presence or absence of 100 µM sucrose with or without 0.1 mM GA for 2 days, and luciferase activity was assayed. + and − indicate the presence or absence of sucrose or GA. The value of luciferase activity in embryos or endosperms in the presence of sucrose but absence of GA was assigned as 1X, and the other values relative to this value were then calculated. The experiments of panels a and b were repeated three times, and error bars indicate the standard deviation of luciferase activity.
activity by an average of 3-fold in sucrose-provided cells and by at least 20-fold in sucrose-starved cells (Fig. 3). The \( \alpha \text{Amy}3 \) SRS also enhanced \( \alpha \text{Act}1 \) promoter activity from a fewfold to tens of fold, depending on the type of tissue and the developmental stage of the transgenic rice assayed (Figs. 5–7). The enhancement was generally effective for all transformants (Fig. 4). One-week-old transgenic rice seedlings contain \(-50\) mM sugars (data not shown). Such a concentration of sugars is high enough to suppress \( \alpha \)-amylase gene expression if it occurs in rice seedlings (4). Consequently, the high activity of \( \alpha \text{Act}1 \)-SRS in transgenic rice is not likely due to sugar starvation. The \( \alpha \text{Act}1 \)-SRS promoter activity is higher than the \( \alpha \text{Act}1 \) promoter activity in both sucrose-provided and sucrose-starved cells (Fig. 3), suggesting that the \( \alpha \text{Amy}3 \) SRS could be a general enhancer regardless of whether sugar is present or not. The consistent effectiveness of the \( \alpha \text{Amy}3 \) SRS in enhancing \( \alpha \text{Act}1 \) promoter activity, as determined by both transient expression and transgenic assays, also suggests that the \( \alpha \text{Amy}3 \) SRS is a faithful transcriptional enhancer for the \( \alpha \text{Act}1 \) promoter. The \( \alpha \text{Act}1 \)-SRS promoter should be of value where a high level of foreign gene expression is required in rice and other monocots. The \( \alpha \text{Act}1 \)-SRS promoter could significantly help in the development of transient expression or stable transformation procedures, in the repression of endogenous gene expression through an antisense RNA or RNA interference approach, in the overproduction of biomolecules or recombinant proteins, and in the expression of disease-, insect-, or stress-resistant genes prior to the employment of nonconstitutive expression strategies.

The \( \alpha \text{Amy}3 \) SRS Enhances the Endogenous Expression Pattern of the \( \alpha \text{Act}1 \) Promoter—The experiments described in both Figs. 5 and 6 show that the activity of the \( \alpha \text{Act}1 \) and \( \alpha \text{Act}1 \)-SRS promoters fluctuates during the development of transgenic rice. The promoter activities reached their first peak within 1 week after germination, declined at week 2 through week 3, and rose again at week 4. The activity profile of the \( \alpha \text{Act}1 \)-SRS was similar to that of the wild-type \( \alpha \text{Act}1 \) promoter in almost every tissue throughout their developmental stages, suggesting that the \( \alpha \text{Amy}3 \) SRS led primarily to an enhancement of the endogenous expression pattern rather than to constitutive ectopic expression. Interestingly, the impact of the \( \alpha \text{Amy}3 \) SRS on the enhancement of the \( \alpha \text{Act}1 \) promoter activity varied from tissue to tissue. The greatest impact occurred in roots, regardless of the developmental stage of the transgenic rice, and the least impact occurred in the endosperms of germinated seeds. The \( \alpha \text{Amy}3 \) SRS activity in rice suspension cells is enhanced by sugar starvation. Whether or not the enhancement potential of \( \alpha \text{Amy}3 \) SRS is regulated by sugar levels in other plant tissues remains to be determined.

Cytoplasmic actin is an essential component of the eukaryotic cell cytoskeleton and plays an important role in various plant cellular activities and extension growth (28, 29). Therefore, it is reasonable to suspect that the \( \alpha \text{Act}1 \) promoter is more active in rapidly growing cells and tissues than in slowly growing cells and tissues. Maximal actin mRNA levels have been observed in the young shoots of rice between 2–4 days old; however, the level began to decline 7 days after germination (30). The interval between leaf production on the main culm of rice is shorter during early growth stages (4–5 days after germination) and longer at later stages (8–9 days after germination) (31). We normally observed that active tiller (side shoots produced at the base of a stem) growth in rice plants begins at weeks 3 and 4. These studies indicate that the activity of the \( \alpha \text{Act}1 \) and \( \alpha \text{Act}1 \)-SRS promoters in transgenic rice seedlings correlates well with seeding vigor, the endogenous actin gene expression pattern, and the growth rate of transgenic rice. This notion is further supported by the observation that \( \alpha \text{Act}1 \) and \( \alpha \text{Act}1 \)-SRS promoter activities are higher in seedlings growing vigorously and healthily under light/dark cycle conditions than in seedlings growing poorly under continuous darkness (Fig. 7). The unique feature of the \( \alpha \text{Amy}3 \) SRS in enhancing an endogenous expression pattern rather than inducing constitutive ectopic expression of a monocot promoter is potentially useful for the generation of activation-tagged mutants of monocots.

The \( \alpha \text{Amy}3 \) SRS and \( \alpha \text{Amy}8 \) SRS/GARS Contain cis-acting Elements for Differential Expression in Rice—\( \alpha \text{Amy}3 \) and \( \alpha \text{Amy}8 \) are the two most abundantly expressed \( \alpha \)-amylase genes in sucrose-starved rice suspension cells, and the levels of \( \alpha \text{Amy}3 \) and \( \alpha \text{Amy}8 \) mRNAs constitute 60 and 30%, respectively, of total \( \alpha \)-amylase mRNAs accumulated in sucrose-starved rice suspension cells (10). The positive correlation between the transcription rates and steady-state mRNA levels of individual \( \alpha \)-amylase genes suggests that transcriptional regulation plays an important role in the differential expression of \( \alpha \)-amylase genes in sucrose-starved rice suspension cells (10). The 105-bp \( \alpha \text{Amy}3 \) SRS and 230-bp \( \alpha \text{Amy}8 \) SRS/GARS share conserved and distinct sequences (Fig. 1). The \( \text{G} \) box and TATCCA element are essential for the \( \alpha \text{Amy}3 \) SRS activity in rice protoplasts under sugar starvation (11). These two elements are also present in the \( \alpha \text{Amy}8 \) SRS/GARS and are likely responsible for the sugar responsiveness of \( \alpha \text{Amy}8 \) SRS/GARS in transgenic embryos (Fig. 11). The \( \alpha \text{Amy}3 \) SRS contains two tandem repeats of the TATCCA element (Fig. 1a), which may be essential for the high activity of the \( \alpha \text{Amy}3 \) promoter. Recently, we found that modification of the \( \alpha \text{Amy}8 \) SRS/GARS by duplicating the TATCCA element also enhances the \( \alpha \text{Amy}8 \) SRS/GARS activity in rice embryos by 6-fold.\(^2\) The \( \text{G} \) box is also essential for the activity of the \( \alpha \text{Amy}3 \) SRS under sugar starvation (11); however, the \( \text{G} \) box is absent in the \( \alpha \text{Amy}8 \) SRS/GARS (Fig. 1). It remains to be determined whether the \( \text{G} \) box provides additional functions for the regulation of \( \alpha \text{Amy}3 \) expression in rice.

The endogenous \( \alpha \text{Amy}3 \) was not at all or only lowly expressed in the embryo, endosperm, shoots, and roots of germinated rice seeds (Fig. 8; see also Ref. 3). We also observed that a 1.1-kb \( \alpha \text{Amy}3 \) promoter sequence directed only a very low level of luciferase expression in these four tissues (data not shown). It is possible that the \( \alpha \text{Amy}3 \) promoter does not contain cis-acting elements capable of controlling gene expression in the four tissues or that a repressor inhibits the \( \alpha \text{Amy}3 \) promoter activity. Interestingly, the \( \alpha \text{Amy}3 \) SRS enhanced \( \alpha \text{Act}1 \) promoter activity significantly in the embryo, shoot, and root (Fig. 9) and weakly in the endosperm (Figs. 5 and 9) of germinated transgenic rice seeds. This suggests that, if there is a repressor, the repressor does not act on the 105-bp \( \alpha \text{Amy}3 \) SRS. Although it is likely that the \( \alpha \text{Amy}3 \) SRS mainly functions as an enhancer, the possibility that the \( \alpha \text{Amy}3 \) SRS contains cis-acting elements capable of controlling gene expression in the four tissues was not ruled out by the present study. On the other hand, the \( \alpha \text{Amy}8 \) SRS/GARS enhanced \( \alpha \text{Act}1 \) promoter activity mainly in the endosperm and embryo of germinated rice seeds (Fig. 9). Fusion of the \( \alpha \text{Amy}8 \) SRS/GARS with the CaMV35S minimal promoter also led to endosperm- and embryo-specific expression of luciferase (Fig. 10), which is consistent with the embryo- and endosperm-specific expression pattern of the endogenous \( \alpha \text{Amy}8 \) (Fig. 8). These results suggest that the 230-bp \( \alpha \text{Amy}8 \) SRS/GARS contains cis-acting elements sufficient for directing endosperm- and embryo-specific expression of promoters. Future functional studies of a linker scan-

---

\(^2\) P.-W. Chen and S.-M. Yu, unpublished results.
mutagenized \(\alpha\text{Amy8} \) SRS/GARS may lead to identification of these cis-acting elements responsible for tissue-specific expression.

Cross-talk between the Sugar and GA Signaling Pathways—The \(\alpha\text{Amy8} \) SRS/GARS contains a putative GARE that might be responsible for the GA inducibility of this promoter sequence in both rice embryos and endosperms. The \(\alpha\text{Amy8} \) SRS/GARS-CaMV35S chimeric promoter conferred high luciferase activity in rice embryos in the absence of both sucrose and GA, but the addition of GA further enhanced the luciferase activity by only 1.5-fold (from 5.5-fold to 8.2-fold) (Fig. 11). One possible explanation for the small increase in luciferase by GA is that both sugar starvation and a saturating level of endogenous GA had already activated the \(\alpha\text{Amy8} \) SRS/GARS in the rice embryos. Consequently, the addition of exogenous GA did not lead to a significant increase in luciferase activity. Barley embryos have been shown to contain endogenous GA that activates \(\alpha\text{Amy8} \) SRS/GARS in barley aleurone layers un-

SRS/GARS may lead to identification of patterns, whereas the \(\alpha\text{Amy8} \) SRS/GARS contains cis-acting elements that function as sugar-sensitive, GA-dependent, and tissue-specific transcriptional enhancers in transgenic rice. In the future, more detailed studies on the essential cis-acting elements present in these enhancers may facilitate the design of highly active and tightly regulated composite promoters for special applications in plant transformation and gene expression in monocots. The present study provides a system for analyzing the molecular mechanisms of differential GA- and sugar-dependent gene regulation and cross-talk between the sugar and GA signaling pathways in cereals.

Acknowledgments—We thank Lin-Tze Yu for technical assistance and Douglas Platt for help in preparation of the manuscript.

REFERENCES
1. Jacobsen, J. V., Gubler, F., and Chandler, P. M. (1995) in Plant Hormones: Physiology, Biochemistry, and Molecular Biology. (Davies, P. J., ed.) pp. 247–271, Kluwer Academic Publishers, Norwell, MA
2. Yu, S. M., Kuo, Y. H., Sheu, G., Sheu, Y. J., and Liu, L. F. (1991) J. Biol. Chem. 266, 21311–21317
3. Yu, S. M., Tsou, W. S., Lo, W. S., Kuo, Y. H., Lee, H. T., and Wu, R. (1992) Gene (Amst.) 122, 247–253
4. Yu, S. M., Lee, Y. C., Fang, S. C., Chao, M. T., Hwa, S. P., and Liu, L. F. (1996) Plant Mol. Biol. 30, 1277–1289
5. Perata, P., Matsukura, C., Vernieri, P., and Yamaguchi, J. I. (1997) Plant Cell 9, 2197–2208
6. Yu, S. M. (1999) in Molecular Biology of Rice (Shimamoto, K., ed.) pp. 161–178, Springer-Verlag, Tokyo
7. Yu, S. M. (1999) Plant Physiol. 121, 687–693
8. Shan, C. T., Chao, Y. C., and Yu, X. M. (1994) J. Biol. Chem. 269, 17635–17641
9. Sheu, J. J., Jan, S. P., Lee, H. T., and Yu, S. M. (1994) Plant J. 3, 655–664
10. Sheu, J. J., Yu, T. S., Tong, W. F., and Yu, S. M. (1996) J. Biol. Chem. 271, 26999–27004
11. Lu, C. A., Lin, E. K., and Yu, S. M. (1998) J. Biol. Chem. 273, 10120–10131
12. Gubler, F., and Jacobsen, J. V. (1992) Plant Cell 4, 1435–1441
13. Lanahan, M. B., Ho, T. H., Rogers, S. W., and Rogers, J. C. (1992) Plant Cell 4, 203–211
14. Zhang, W., McElroy, D., and Wu, R. (1991) Plant Cell 3, 1155–1165
15. Christensen, A. H., Sharrocks, R. A., and Quail, P. H. (1992) Plant Mol. Biol. 18, 675–689
16. McElroy, D., and Brettell, R. I. S. (1994) Trends Biotechnol 12, 62–68
17. Schledzewski, K., and Mendel, R. R. (1994) Transgenic Res 3, 249–255
18. Wilmink, A., van de Ven, B. C., and Duns, J. J. (1995) Plant Mol. Biol. 28, 949–955
19. Olive, M. R., Walker, J. C., Singh, K., Dennis, E. S., and Peacock, W. J. (1996) Plant Mol. Biol. 15, 593–604
20. Ellis, J. G., Llewellyn, D. J., Walker, J. C., Dennis, E. S., and Peacock, W. J. (1997) EMBO J. 6, 2153–2162
21. Thomas, B. R., and Rodriguez, R. L. (1994) Plant Physiol. 106, 1235–1239
22. Schuller, K., and Mendel, R. R. (1994) Transgenic Res 3, 249–255
23. Christensen, A. H., Sharrocks, R. A., and Quail, P. H. (1992) Plant Mol. Biol. 18, 675–689
24. Last, D. I., Brettell, R. I. S., Chamberlain, D. A., Chaudhury, A. M., Larkin, P. J., Marsh, E. L., Peacock, W. J., and Dennis, E. S. (1991) Theor. Appl. Genet. 81, 581–588
25. Straiter, J. J., and Schilperoort, M. (1987) Prototetras 141, 1–12
26. Seagull, R. W. (1989) Cell 58, 167–177
27. Xiong, X., Xu, C., and Qi, L. (1999) J. Biol. Chem. 274, 1155–1161
28. Vergara, B. S. (1980) in Rice: Production and Utilization (Lah, B. S., ed.) pp. 75–86, AVI Publishing Co., Inc., Westport, CT
29. Loreti, E., Matsukura, C., Gubler, F., Alpi, A., Yamaguchi, J., and Perata, P. (2000) Plant Mol. Biol. 44, 85–90
30. Gubler, F., Kalla, R., Roberts, J. K., and Jacobsen, J. V. (1999) Plant Cell 17, 1–9
31. Uhmann, R., Perata, P., Putsuhara, Y., and Yamaguchi, J. (1998) Planta 204, 429–428
32. Last, D. I., Brettell, R. I. S., Chamberlain, D. A., Chaudhury, A. M., Larkin, P. J., Marsh, E. L., Peacock, W. J., and Dennis, E. S. (1991) Theor. Appl. Genet. 81, 581–588
33. Straiter, J. J., and Schilperoort, M. (1987) Prototetras 141, 1–12
34. Seagull, R. W. (1989) CRC Rev. Plant Sci. 8, 131–167
35. McElroy, D., Rothenberg, M., Reece, K. S., and Wu, R. (1990) Plant Mol. Biol. 15, 257–288
36. Vergara, B. S. (1980) in Rice: Production and Utilization (Lah, B. S., ed.) pp. 75–86, AVI Publishing Co., Inc., Westport, CT
37. Oreii, E., Matsukura, C., Gubler, F., Alpi, A., Yamaguchi, J., and Perata, P. (2000) Plant Mol. Biol. 44, 85–90
38. Gubler, F., Kalla, R., Roberts, J. K., and Jacobsen, J. V. (1999) Plant Cell 17, 1–9
39. Cesaris, M., Gomes-Cadenas, A., and Ho, T. H. (1999) Plant Cell 11, 107–118
40. Gomes-Cadenas, A., Zentella, R., Walker-Simmons, M. K., and Ho, T. H. (2001) Plant Cell 13, 667–679
41. Sheu, J. J., and Chen, S. A. (1999) J. Biol. Chem. 274, 1155–1161
42. Gibson, S. I. (2000) Methods Enzymol. 316, 107–181
43. Hood, E. E., Helmer, G. L., Fraley, R. T., and Chilton, M. D. (1986) J. Bacteriol. 168, 1291–1301

3 C.-A. Lu, T.-H. D. Ho, and S.-M. Yu, submitted for publication.
Rice α-Amylase Transcriptional Enhancers Direct Multiple Mode Regulation of Promoters in Transgenic Rice
Peng-Wen Chen, Chung-An Lu, Tien-Shin Yu, Tung-Hi Tseng, Chang-Sheng Wang and Su-May Yu

J. Biol. Chem. 2002, 277:13641-13649.
doi: 10.1074/jbc.M109722200 originally published online February 6, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M109722200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 41 references, 16 of which can be accessed free at http://www.jbc.org/content/277/16/13641.full.html#ref-list-1