Promoter elements required for sugar-repression of the R\(\alpha\)my3D gene for \(\alpha\)-amylase in rice

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Abstract There is increasing evidence showing that cereal \(\alpha\)-amylase gene expression is controlled not only by the classical hormonal regulation, but also by feed-back sugar repression. We demonstrated by in situ hybridization that the sugar repression of rice \(\alpha\)-amylase gene R\(\alpha\)my3D takes place in scutellar epithelium cells of callus-forming rice embryos. We also used a transient expression system to study the cis-acting elements involved in the sugar repression of the R\(\alpha\)my3D promoter activity. Site-directed mutagenesis of the 50-bp nucleotide sequence from −172 to −123 revealed that consensus sequences of G motif (TACGTA) and TATCCA T/C motif (GATA motif as its antisense sequence) are responsible for sugar repression. The promoter sequences required for sugar repression are reported and discussed.

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Key words: cis-acting element; Scutellar epithelium; G motif; GATA motif; Sugar sensing; Oryza sativa

1. Introduction

During germination of cereal grains, \(\alpha\)-amylases (EC 3.2.1.1) play a key role in the mobilization of the energy reserves constituted by insoluble starch granules ([1] for review). The enzyme synthesized during the germination of cereal seeds catalyzes the hydrolysis of the \(\alpha\)-1,4 glucan bonds of the starch molecule. It is commonly accepted that, even though other amylolytic enzymes participate in the process of starch breakdown, the contribution of \(\alpha\)-amylase is the prerequisite for the initiation of this process ([2] for references).

\(\alpha\)-Amylase is not present in the dry cereal seed, but is rapidly induced by the action of gibberellins (GAs), produced by the embryo, triggering \(\alpha\)-amylase gene regulation in the aleurone layers. The induction of \(\alpha\)-amylase by GA in cereal grains and the counteractive role of ABA on the same process represent a classical model system for studying the mode of hormonal regulation, but also by feed-back sugar repression. We demonstrated by in situ hybridization that the sugar repression of R\(\alpha\)my3D gene expression system to study the cis-acting elements for the activity of the R\(\alpha\)my3D promoter. Our data indicate that consensus nucleotide sequence of G and GATA motifs are important for the sugar repression of the gene.

2. Materials and methods

2.1. Preparation of rice embryos

Rice seeds (Oryza sativa, cv. Notohikari) were sown in petri dishes containing liquid Murashige-Skoog salt mixture and 2 mg/l 2,4-D. Seeds germinated on this medium show enlarged scutellar sides (where \(\alpha\)-amylase is expressed in vivo, see Fig. 2), allowing an accurate targeting of the gold particles into the scutellum. All the subsequent procedures were performed as described by Umemura et al. [8].

2.2. Chimeric gene constructs

Using the polymerase chain reaction technology, HindIII and XhoI restriction endonuclease sites were created at the 5′ flanking region (−422, −222, −172 and −122 to −65) of the R\(\alpha\)my3D gene from the rice genomic clone (Osg1A). The nucleotide sequence and other characteristics of the gene have been reported before [13]. The amplified promoter was attached using the HindIII and XhoI restriction endonuclease sites of a truncated minimal (−46) cauliflower mosaic virus (CaMV) 3SS promoter to the sequence coding for the Escherichia coli \(\beta\)-glucuronidase (gusA) gene with a modified ATG initiation codon. The first intron from the castor bean catalase gene was inserted into the 5′ untranslated sequence [14]; this construct (R\(\alpha\)my3D promoter−46 of CaMV 3SS promoter/first intron of catalase gene/gusA) is identified as R\(\alpha\)my3D-GUS. As an internal standard, we used the 3SS-LUC clone (pREX\(\beta\)LUC), a construct of the 3SS promoter, \(\Omega\) sequence and first intron of a gene for phaseolin fused with luciferase gene (LUC) [15], a gift from Dr. Hirochika. The 3SS-LUC construct expression in rice embryo was unaffected by the sugars and other chemicals used in our experiments.

2.3. Transient expression system

Unless otherwise indicated, all experiments were performed with particle-bombardment co-delivery of R\(\alpha\)my3D-GUS and 3SS-LUC for data normalization described by Umemura et al. [8].

Extraction and assays of samples for GUS and LUC activities were performed as described by Lanahan et al. [16] but incubations for GUS assays were 1 h long. Typical LUC activities were in the range 200000–500000 RLU (relative light units) (background from non-transformed tissue was 50–100 RLU). In order to allow easy comparison of the data presented in the different figures, data were expressed as ‘relative GUS/LUC activity (%)’ with respect to the control (relative activity = 100).

2.4. Sugar assay

Extraction of plant material, recovery experiments and sugar assays for sucrose, glucose and fructose were performed as an enzyme coupling method to monitor the reduction of NAD [17].

2.5. In situ hybridization

Callus-forming rice embryos were fixed in FAA (formalin/acetic acid/50% ethanol (1:1:18)) for 24 h at 5°C. After dehydration in a graded 2-methyl-2-propanol series, samples were embedded in PARA-
PLAST (Oxford Labware, St. Louis, MO, USA) and sectioned at 10 μm by rotary microtome, and applied on slide glasses treated with 3-aminopropyltrichlorosilane (Shinetsu Chemicals, Tokyo, Japan). Digoxigenin-labeled RNA probe was prepared from the coding region of rice α-amylase gene, RAmy3D cDNA clone (pOS137). Probes were degraded to a mean length of 150 bp by incubating in alkali at 60°C. In situ hybridization was performed according to Kouchi and Hata [18]. Hybridization signals were detected according to Kouchi and Hata [18]. Hybridization signals were not detected when sense probes were used. Accordingly, only results obtained using the antisense probe were shown.

3. Results

3.1. RAmy3D gene transcript is exclusively expressed in sugar-depleted scutellar epithelium cells of rice embryo

It is well known that α-amylase activity in cereal seeds is detectable in the scutellar epithelium of the embryo and in the aleurone layer of the endosperm [19]. In situ hybridization techniques revealed that mRNA for rice α-amylase gene RA-my1A, a GA inducible high p group gene, is initially detected in the scutellar epithelium and appeared in the aleurone layer at a later stage of germination [3,20]. The rice α-amylase gene RAmy3D is known to be induced under sugar starvation conditions but not by GA in the isolated embryos and suspension cultured cells of rice [4–6,8,21].

Rice embryos excised from the endosperm of germinating seedlings contain their own carbohydrates. Among them, glucose (arising from starch degradation in the endosperm), sucrose (synthesized in the scutellum), and fructose (derived from sucrose degradation) can down-regulate the RAmy3D gene expression. We measured the levels of sucrose, fructose and sucrose in the callus-forming embryos after excision from the endosperm (condition 1 in Fig. 1) as well as after 1- and 3-day incubation of the excised embryos on sugar-free medium (i.e. sugar starvation treatment, conditions 2 and 3) and on medium containing 90 mM glucose for 2 days after 1-day sugar starvation (condition 4). The results showed that rice embryos contain sucrose, glucose and a relatively lower level of fructose, and that incubation on the sugar-free medium rapidly leads to a decrease in the endogenous content of sucrose and, mainly, glucose.

We performed in situ hybridization of callus-forming rice embryos with an antisense probe to demonstrate the location and timing of the sugar repression of R Amy3D gene (Fig. 2). The hybridization signal for the R Amy3D mRNA cannot be detected in the embryos immediately after excision from the endosperm (Fig. 2A), where the cells show a high level of endogenous carbohydrate (see condition 1 in Fig. 1). Instead we were able to detect the signal in the embryos after 1- or 3-day sugar starvation treatment (Fig. 2B,C). The activation of the mRNA transcription was reversibly suppressed by 2-day glucose treatment after 1-day starvation (Fig. 2D). Magnified figures revealed that the signal for R Amy3D transcript is detectable in the outer-surface cell layers of the embryo (Fig. 2E) which are originally derived from the scutellum epithelium. The signal is completely repressed in newly divided cells under sugar-rich conditions (Fig. 2F). Interestingly, the signal was also detected in the vascular cells under sugar starvation (Fig. 2C). Starch granules visualized by PAS staining completely disappeared in the cells under sugar starvation (Fig. 2G), whereas they developed again after glucose treatment (Fig. 2H), indicating a good correlation between sugar starvation and disappearance of starch granules.

![Fig. 1. Endogenous carbohydrate content in callus-forming rice embryos. A: glucose; B: fructose; and C: sucrose. Five embryos excised from the endosperm (condition 1) were incubated on sugar-free medium for 1 day (condition 2). After that starvation, embryos were incubated on sugar-free medium for additional 2 days (condition 3) or on medium containing 90 mM glucose for 2 days (condition 4). Data are means ± S.E. (n = 3).](image)

3.2. Sugar-repressive cis-acting elements of R Amy3D promoter are involved in consensus sequences of G motif and TATCCA TC motif

Addition of carbohydrates to the incubation medium of transformed rice embryos resulted in repression of the R Amy3D promoter activity (Fig. 3, –422 to –65 construct). Embryos were dissected from seedlings, transformed with a 5’ deleted promoter of R Amy3D-GUS co-delivered with 35S-LUC by particle bombardment, and transferred to sugar-free medium (control), or to medium containing 90 mM sucrose for 2 days. As shown in Fig. 3A, the R Amy3D promoter activity visualized by GUS staining is repressed by sugar. While deletion of the –422 to –172 sequence had no effect on the promoter activity, deletion of the –422 to –122 fragment resulted in a dramatically reduced promoter activity under sugar starvation conditions (control of –122 to –65 in Fig. 3B). These results from the 5’ deletion analyses suggest that the 50-bp nucleotide sequence from –172 to –123 of the R Amy3D promoter is responsible for the sugar repression.

Experiments were performed using R Amy3D promoters after mutagenesis of the 50-bp nucleotide sequence from –172 to –123 (Fig. 4). Mutagenesis of 8-bp sequences at –161 to –154 (M2), –151 to –144 (M3) and –131 to –124 (M5) results in a significant reduction of the promoter activity under sugar starvation, whereas it had no effect at –171 to –164 (M1) and –141 to –134 (M4). These results suggest that the sequences at –161 to –144 (M2 and M3) and –131 to –124 (M5) are involved in the sugar-responsive cis-acting elements. From a comparison between those sequences and registered cis-acting motif sequences on the data base, we found consensus sequences designated as G motif (consensus CACGTG, –154TACGTG—149 for R Amy3D) and TATCCA TC motif (–131TATCCAT–125) (Fig. 5). Both nucleotide sequences might therefore be important for the expression of the R Amy3D gene under sugar starvation.

4. Discussion

4.1. Sugar repression of α-amylase gene expression

Rice α-amylase genes, R Amy3D and 3E (also Amy3D and
Amy3E) are mainly under sugar control, with phytohormones playing little if any role [6]. We demonstrated by in situ hybridization that the sugar repression of rice α-amylase gene RAmy3D takes place in scutellar epithelium cells of callus-forming rice embryos (Fig. 2). Although the sugar repression has been thought to be restricted to the 3D and 3E genes [6,22], the RAmy1A gene, which is clearly under hormonal control in the aleurone [23], is also affected by sugar regulation but to a smaller extent when compared with that of RAmy3D [11]. Furthermore, Perata et al. [12] have reported that even barley α-amylase genes (both high and low pf group) are under sugar control in the embryo (but not in the aleurone), indicating that α-amylase, a key enzyme of starch degradation in cereal seedlings, may be generally under sugar control in the embryos.

4.2. Sugar-repressive cis-acting elements in α-amylase and other plant genes

Preliminary promoter characterization of the RAmy3D gene using transgenic cell cultures of rice have been reported by Huang et al. [7]. DNase I footprinting analyses using binding activity of a nuclear protein from the suspension-cultured cells...
of rice to the RAmy3D promoter sequence revealed three protein-binding regions. Each of these protein-binding sequences contained the GCCCG G/C CG motif [24]. These heptameric binding motifs are located at −269, −243 and −209 of the gene. From the present demonstrations, however, those binding motifs do not seem to be critical for the sugar repression of the RAmy3D gene. Site-directed mutagenesis of 8-bp sequences at −161 to −154 (M2), −151 to −144 (M3) and −131 to −124 (M5) results in a significant reduction of the promoter activity under sugar starvation (Fig. 4), indicating that those nucleotide sequences are associated with sugar repression. Finally we found consensus sequences designated as G motif (consensus CACGTG, 3161TACGTG 3149 for RAmy3D) and TATCCA T/C motif (3131TATCCAT 3125) in the regions. Both motif sequences are probably important for the expression of the RAmy3D gene under sugar starvation. G motif, however, could not be found in the promoter sequence of RAmy1A around the position, although the gene is also under sugar control, whereas TATCCA T/C motif can be found in both the promoter sequences at the same position (see Fig. 5). TATCCA T/C motif contains GATA motif as its antisense sequence, especially TATCcaTATC sequence for RAmy3D means GATAtgGATA sequence as its antisense sequence, which has been reported in petunia cab22L gene promoter as a light-regulated motif [25]. Instead of the G motif for the RAmy3D, GARE (TAACAAA) can be found in the RAmy1A, which is well characterized as a GA-responsive cis-acting element [16,26–28] (Fig. 5).

Hwang et al. (1998) recently reported the cis-elements required for rice α-amylase Amy3D (identified as RAmy3D in this experiment) expression during sugar starvation [29]. Their functional promoter analyses using electroporated rice protoplasts revealed that three sequences having the greatest effects on Amy3D gene expression included the CGACG element and additional two cis-elements that are reported in this experiment, i.e. the amylase element (TATCCA T/C motif) and G box-related element. These compatible results strongly suggest the specific sugar-repressive cis-elements of the RAmy3D pro-
moter. For the first CGACG element, however, the site-directed mutagenesis (M1) in this experiment (tagCG from CGACG, see Fig. 4) showed no reduction of the promoter activity under sugar starvation, indicating that distinct experimental systems (protoplasts vs. embryos) may lead to diverse results.

Three conserved sequences for the promoter region of most GA-inducible α-amylase genes in cereals have been reported, i.e. pyrimidine motif (CCTTTT) at diverse positions, GARE (gibberellin response element) for RAm1A; TAACAAA, GARE (gibberellin response element) for RAm3D; TAAACCA, GARE (gibberellin response element) for RAm1A. For specific characters for these motifs, see in the text.

4.3. G motif and GATA motif as a sugar-response cis-acting element

G motif is a hexameric motif, CACGTG, found in many diverse plant genes. This sequence functions as a cis-acting promoter element, and is first characterized on the 5' flanking region of the light-regulated ribulose 1,5-bisphosphate small subunit (RBcS) genes. After this report, many groups have shown that the G motif sequence resides in the promoters of many genes that are switched on in response to quite diverse stimulatory pathways, i.e. light, anaerobiosis, p-coumaric acid and phytohormones such as abscisic acid, ethylene and methyl jasmonate ([35] for review). Our results from functional promoter analyses of rice α-amylase gene, RAm1A3D, suggest that a G motif-like element also responds to endogenous carbohydrate levels (Fig. 4). In each of the promoters reported previously, the G motif resides in a unique DNA context and additional elements are critical to the appropriate response. In the case of RAm1A3D gene, additional GATA motif sequences may be critical to the specific sugar response. GATA (or J) motif has also been identified as a light-regulated cis-acting element ([25] for review). Indeed, detailed experiments by Puente et al. [36] indicate that combination of G and GATA motifs can serve as minimal autonomous promoter determinants which integrate light and developmental signals and modulate promoter activity. The cellular level of carbohydrates, end product of photosynthetic function, is probably the trigger signal(s) involved in light regulation. Indeed the expression of most photosynthesis genes is regulated by metabolizable carbohydrates [37]. Further study will be needed to test this speculation.

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