Functional dissection of a sugar-repressed α-amylase gene (*RAmy1A*) promoter in rice embryos

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Received 12 December 1997

Abstract The gibberellin-inducible rice α-amylase gene, *RAmy1A*, was demonstrated to be sugar repressed in rice embryos and functional dissection of the promoter of *RAmy1A* in relation of its sugar-modulated expression was performed. Gibberellin-response *cis*-elements of GARE (TAACAAA) and pyrimidine box (CCTTTT) were partially involved in the sugar repression.

Key words: Sugar repression; *cis*-acting element; Gibberellin; Glucose; α-Amylase gene; Rice (*Oryza sativa*)

1. Introduction

The production of α-amylase during germination of cereal seeds plays a crucial role in providing growing seedling with metabolizable carbohydrates. It is commonly accepted that, even thought other amylolytic enzymes participate in the process of starch breakdown, the contribution of α-amylase is the prerequisite for the initiation of this process [1].

α-Amylase is not present in the dry cereal seed, but is rapidly induced by the action of gibberellic acid, produced by the embryo, triggering α-amylase gene regulation in the aleurone layers. Beside the aleurone layers, also the scutellum plays an important role in the production of α-amylase, although it is still not known if its induction is hormone dependent.

There is increasing evidence showing that in rice α-amylase gene expression is controlled not only by the classical hormonal regulation, but also by feed-back metabolite repression. However, in rice, only two of the ten genes encoding for α-amylase isoforms [2,3] appear to be strongly under the control of sugar level, namely *RAmy3D* and *RAmy3E* (also identified as αAmy3 and αAmy8) [4–9]. While the expression of the *RAmy3D* gene is restricted to the embryo, the *RAmy1A* (αAmy7) gene is expressed in both the embryo and aleurone layers. In the aleurone layers, the *RAmy1A* gene is under hormonal control [10]. Regulation of the *RAmy1A* by sugars has not been reported, but a recent paper by Sheu et al. [11] demonstrates that also the *RAmy1A* gene is affected by sugar availability in rice suspension cultures. More recently, we have shown that GA-induced α-amylase genes in barley are sugar repressed [12]. In this paper we demonstrated that the *RAmy1A* gene is sugar repressed in rice embryos, and performed a functional dissection of the *RAmy1A* promoter in relation of its sugar-modulated expression in the embryo.

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 side (where α-amylase is expressed in vivo), allowing an accurate targeting of the gold particles into the scutellum. All the subsequent procedures were performed as described by Umemura et al. [9].

2.2. Chimeric gene constructs

Using the polymerase chain reaction technology, *Hin*dIII and *Xho*I restriction endonuclease sites were created at the 5′ flanking region (−748, −228, −175 and −141 to −46) of the *RAmy1A* gene from the rice genomic clone (λOSg2). The nucleotide sequence and other characteristics of the gene have been reported before [2]. The amplified promoter was attached using the *Hin*dIII and *Xho*I restriction endonuclease sites of a truncated minimal (−46) cauliflower mosaic virus (CaMV) 35S promoter to the sequence coding for the *Escherichia coli* β-glucuronidase (GUS) gene with a modified ATG initiation codon. The first intron from the mung bean catalase gene was inserted into the 5′ untranslated sequence [13]; this construct (*RAmy1A* promoter/−46 of CaMV 35S promoter/first intron of catalase gene/gusA/pUC19) is identified as *RAmy1A-GUS*. *RAmy3D-GUS* was also constructed as described by Umemura et al. [9]. As internal standard, we used the 35S-LUC clone (pREXΦLUC), a construct of the 35S promoter fused with luciferase gene (LUC) [14] gifted from Dr. Hirochika (National Institute of Agrobiological Resources, Tsukuba). The 35S-LUC construct expression in rice embryo was unaffected by the sugars and other chemicals used in our experiments (see below).

2.3. Transient expression system

Unless differently stated, all experiments were performed with particle-bombardment co-delivery of *RAmy1A-GUS* or *RAmy3D-GUS* and 35S-LUC for data normalization described by Umemura et al. [9].

Bombardment was performed according to the instruction provided by the manufacturer (BioRad) by using a 1100 psi He pressure and the sample holder closer to the gun (5 cm from the stopping screen). The bombardment was repeated twice on each plate containing 30–40 embryos. After bombardment, embryos were transferred in petri dishes containing the medium above described, eventually supplemented with filtered-sterilized glucose.

Each experiment was repeated two to three times, on different days, and with freshly prepared new batches of reagents and rice embryos. Each independent experiment consisted of three replicates of five embryos each. All repeated experiments gave consistent results. The reported data are means of the obtained results from a representative experiment.

2.4. Enzyme assays

Extraction and assays of samples for GUS and LUC activities were performed as described by Lanahan et al. [15], but incubations for GUS assays were 1 h long. Typical LUC activities were in the range

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Abbreviations: GUS, β-glucuronidase; LUC, luciferase; 2,4-D, 2,4-dichloroacetic acid; GA, gibberellin; ABA, abscisic acid

PII S0014-5793(98)00067-2

200 000–500 000 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).

2.5. Northern blots

RNA extraction was performed by using the aurintricarboxylic acid method as described by Skadsen [16], with minor modifications. Because we extracted RNA from low-starch containing tissues (embryos), the calcofluor step was omitted. Procedures of Northern blots were performed by the standard method as described by Perata et al. [17]. Equal loading was checked by reprobing with a rRNA cDNA probe and ethidium bromide staining. Blots were exposed using a Fujix BAS2000 Bio-Imaging analyzer (Fuji Photo Film Co. Ltd. Tokyo, Japan).

2.6. Sugar assay

Extraction of plant material, recovery experiments and sugar assays for sucrose, glucose and fructose were performed as previously described [17].

3. Results and discussion

3.1. RAmy1A gene transcript level is affected by sugar availability

The transcript of the rice α-amylase gene *RAmy1A* is known to be abundant during seed germination [18]. The *Ramy1A* gene belongs to the GA-inducible high pI group of α-amylases as deduced from DNA sequence analysis, and is identified as isoform A among rice α-amylase proteins [19]. Isoform analysis using suspension cultured cells of rice showed that the expression of *RAmy1A*-encoded protein is not significantly influenced by presence of metabolic sugars, whereas the expression of *RAmy3D*-encoded protein is completely repressed [8]. However a recent report by Sheu et al. [11] suggests regulation of *RAmy1A* gene expression by sugars.

In order to evaluate the sugar-responsiveness of the *RAmy1A* gene, we performed experiments by comparing the effects of sugar starvation, followed by exogenous glucose feeding, on the levels of the *RAmy1A* and *RAmy3D* transcripts (Fig. 1). As expected, the level of the *RAmy3D* mRNA increased rapidly as a consequence of the starvation treatment (Fig. 1B). Exogenous glucose feeding after 1 day of starvation resulted in a reduction of the *RAmy3D* mRNA level: a slight reduction was observed by treating embryos with 10 mM glucose, and a more than 50% reduction was observed in the embryos treated with 30 mM glucose (Fig. 1B). A moderate *RAmy1A* mRNA signal was detected in the control embryos (lane 1 in Fig. 1A), and the mRNA level increased strongly during the starvation treatment. Addition of glucose to the incubation medium resulted in a reduced mRNA level, but only when concentrations higher than 30 mM (30% reduction) were used (lane 5 in Fig. 1A). Indeed, only the 90 mM glucose treatment resulted in a strong reduction of both the *RAmy1A* and *RAmy3D* mRNA levels, consistent with the strong increase in the glucose content of the embryos (Fig. 1D). Beside glucose, the content of sucrose and fructose also increased following feeding with glucose (Table 1), indicating rapid metabolism and interconversion of these carbohydrates, possibly through a cycle of sucrose synthesis/degradation.

Modulation of α-amylase genes by carbohydrates arises from both transcriptional and posttranscriptional processes [11]. We used a transient expression system to compare sugar

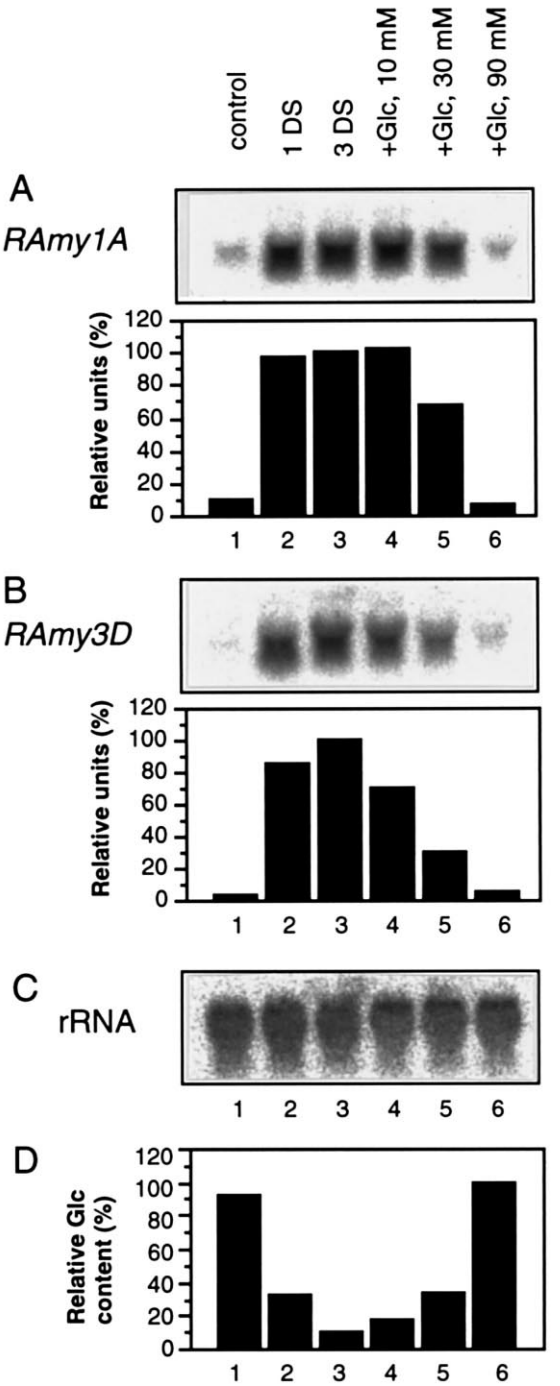

Fig. 1. Effect of glucose on mRNA levels of α-amylase genes (*RAmy1A* and *RAmy3D*) in rice embryos. Embryos dissected from the endosperm (control; lane 1) were incubated for 1 day on a glucose-free medium (1 DS, 1 day starvation; lane 2). After 1 day starvation, embryos were incubated for additional 2 days on a glucose-free medium (3 DS, 3 day starvation; lane 3), or for 2 days on a glucose-containing medium at the concentration of 10 mM (+Glc, 10 mM; lane 4), 30 mM (+Glc, 30 mM; lane 5) and 90 mM (+Glc, 90 mM; lane 6). A: Pattern and quantitation of *RAmy1A* mRNA level. Relative unit is expressed as lane 3: 100. B: Pattern and quantitation of *RAmy3D* mRNA level. C: Pattern of rRNA level. D: Glucose content in rice embryos used in the experiment. Data were derived from a part of Table 1. Relative glucose content is expressed as lane 6: 100.

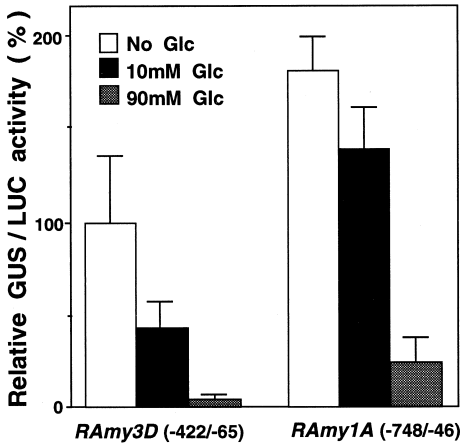

Fig. 2. Effect of glucose on repression of *RAmy1A* and *RAmy3D* promoter activity. Rice embryos treated for 1 day glucose starvation were transformed by bombardment with *RAmy1A* (−748/−46)-*GUS* or *RAmy3D* (−422/−65)-*GUS* co-delivered with 35S-LUC. After transformation the embryos were subsequently incubated for 2 days on glucose-free medium (No Glc) medium containing 10 mM glucose (10 mM Glc) or 90 mM glucose (90 mM Glc). Data were normalized by using the 35S-LUC construct as internal standard. Relative GUS/LUC activity is expressed as: No glc (*RAmy3D*) = 100. Data are means ± S.D. ($n = 3$).

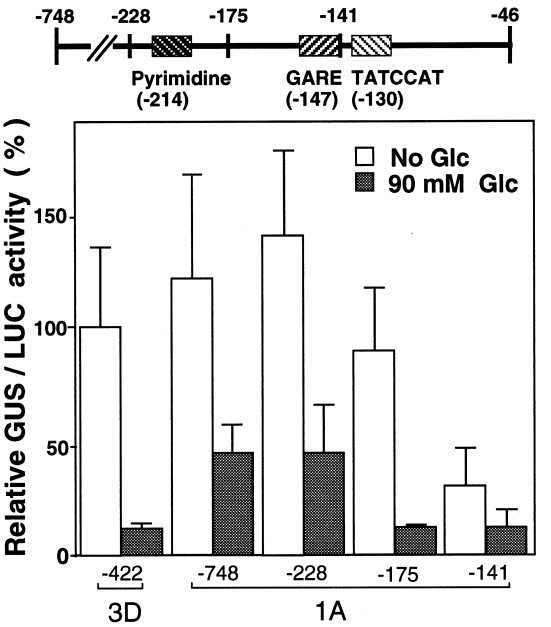

Fig. 3. Effect of 5′ flanking deletions on glucose repression of *RAmy1A* promoter. Schematic diagram of *RAmy1A* promoter was expressed as upper panel. The pyrimidine (−214), GARE (−147) and TATCCAT (−130) sequences are conserved among promoter region of high pI type α-amylase gene in cereals [2]. Rice embryos treated for 1 day on glucose-free medium were transformed by bombardment with 5′ deleted promoter (−748, −228, −175 and −141) of *RAmy1A* (/−46)-*GUS* or *RAmy3D* (−422/−65)-*GUS* co-delivered with 35S-LUC. After transformation the embryos were subsequently incubated for 2 days on glucose-free medium (No Glc), or on medium containing 90 mM glucose (90 mM Glc). Data were normalized by using the 35S-LUC construct as internal standard. Relative GUS/LUC activity is expressed as: No glc (*RAmy3D*) = 100. Data are means ± S.D. ($n = 3$).

repression of the *RAmy1A* and *RAmy3D* genes. The data reported in Fig. 2 show that the promoters of both α-amylase genes are sugar repressed, and that the *RAmy3D* promoter shows a higher sensitivity to relatively low glucose concentrations (10 mM). This indicates that, besides the *RAmy3D* gene, also the *RAmy1A* gene transcription is affected by sugar availability. Differently from the *RAmy3D* gene which is only sugar modulated (hormones play no role), the *RAmy1A* is hormone modulated [10], and its promoter contains the putative *cis*-acting elements involved in GA and ABA modulation. We took advantage of the available data on the characterization of the promoter region of hormone-modulated α-amylase genes to functionally dissect the *RAmy1A* promoter region in relation to its modulation by sugar availability.

3.2. *GA-response cis-elements of RAmy1A promoter are partially involved in sugar repression of the gene*

Gibberellin-responsive *cis*-elements of α-amylase gene have been well characterized using barley aleurone layers. Skriver et al. [20] have reported that the 21-bp sequence contains a conserved motif, TAACAAA, identified as gibberellin-response element (GARE). The roles of this putative GARE and of other closely associated conserved motifs in cereal α-amylase gene promoter (the pyrimidine CCTTTT and TATCCAT motif) have been clarified by Gubler and Jacobsen [21,22], indicating that GARE and TATCCAT/C sequences play an important role in GA-regulated expression. In rice, functional promoter analysis of *RAmy1A* gene using transgenic rice revealed that 5′ flanking region from −232 to +31, which has the three conserved sequences, is sufficient for temporal, spatial and hormonal regulation of the gene expression [10].

Fig. 3 reports the results of 5′ truncated promoter analyses of *RAmy1A* gene. The promoter region was deleted to remove the conserved regions having a putative role in the hormonal regulation of α-amylase genes. Deletion of the −784 to −228 sequence had no effect on the *RAmy1A* promoter activity, while deletion of the −784 to −175 fragment (removing the pyrimidine box) resulted in a reduced promoter activity, but

Table 1
Carbohydrate content in rice embryos incubated on a sugar-free medium or on medium containing various concentrations (10, 30 and 90 mM) of glucose

Treated embryos	Glucose	Fructose	Sucrose
Isolated	15.33 ± 2.47	6.93 ± 0.39	98.39 ± 7.70
1 day starved	5.34 ± 1.99	2.61 ± 1.08	52.27 ± 11.3
43 day starved	1.81 ± 0.35	0.38 ± 0.08	11.84 ± 1.35
1 day starved and 2 day glucose treated (10 mM)	2.82 ± 0.51	1.27 ± 0.11	24.99 ± 1.86
1 day starved and 2 day glucose treated (30 mM)	5.48 ± 0.82	2.56 ± 0.68	62.21 ± 1.99
1 day starved and 2 day glucose treated (90 mM)	16.55 ± 2.28	5.38 ± 1.42	136.26 ± 16.22

Experimental conditions are basically identical to those described in the legend of Fig. 1. Data are expressed as μmol/g fresh weight. Data are means ± S.E. ($n = 3$).

this was not statistically significant. A significant reduction was obtained by deleting the −784 to −141 sequence (removing both the pyrimidine and GARE sequences). Sugar repression was effective on the expression of all the deletion constructs but the −784 to −141, although in this case the observed sugar effect was not statistically significant. These results suggested that the GARE sequence plays a major role in the expression of the *RAmy1A* in rice embryos, and that the elements needed for sugar repression may overlap with those for hormonal regulation.

Experiments were performed using *RAmy1A* promoters after mutagenesis of the three conserved sequences (Fig. 4). Mutagenesis of the pyrimidine and GARE sequences results in a significant reduction of the promoter activity. Both elements are therefore important for the expression of the *RAmy1A* promoter in rice embryos. On the other hand, the TATCCAT box revealed to be not needed for the *RAmy1A* promoter activity. Sugar repression was observed with all the constructs used, indicating that it is unlikely that sugar repression is triggered by the interaction of a *trans*-acting factor on a specific sequence of the *RAmy1A* promoter. Indeed, both the GARE and pyrimidine sequences are not conserved in promoter region of *RAmy3D* gene. Overall the results might indicate that sugar repression of *RAmy1A* gene expression is not due to a specific *cis*-sequence of the promoter but is instead consequence of the sugar signalling process(es) upstream of the transactivation of the *RAmy1A* gene. Perata et al. [12] recently reported that sugar and hormonal signalling interact

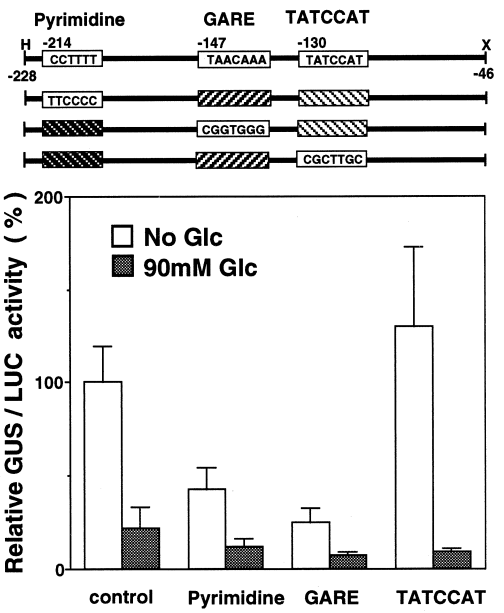

Fig. 4. Effect of site-directed mutagenesis of three conserved sequences on glucose repression of *RAmy1A* promoter. Diagram showing native and mutated sequences at the position of pyrimidine (−214), GARE (−147) and TATCCAT (−130) sequences is shown in the upper panel. Rice embryos treated for 1 day on glucose-free medium were transformed by bombardment with native (control) and mutagenized *RAmy1A* (−228/−46)-*GUS* and co-delivery of 35S-LUC. After transformation the embryos were subsequently incubated for 2 days on glucose-free medium (No Glc), or on medium containing 90 mM glucose (90 mM Glc). Data were normalized by using the 35S-LUC construct as internal standard. Relative GUS/LUC activity is expressed as: No glc (control) = 100. Data are means ± S.D. ($n = 3$).

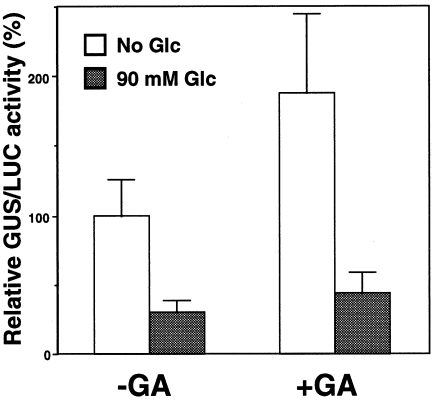

Fig. 5. Effect of GA_3 on glucose repression of *RAmy1A* promoter. Rice embryos treated for 1 day on glucose-free medium were transformed by bombardment co-delivery with *RAmy1A* (−228/−46)-*GUS* and co-delivery of 35S-LUC. After transformation the embryos were subsequently incubated for 2 days on glucose-free medium (No Glc), or on medium containing 90 mM glucose (90 mM Glc). As gibberellin treatment, 5 μl of 10 μM GA_3 were applied to each embryo after transformation. Data were normalized by using the 35S-LUC construct as internal standard. Relative GUS/LUC activity is expressed as: No glc (−GA) = 100. Data are means ± S.D. ($n = 3$).

in the regulation of gibberellin-induced α-amylase gene expression in barley embryos.

The repression of *RAmy1A* gene expression triggered by sugars is independent from an osmotic effect, since a mannitol treatment (90 mM) was unable to repress the promoter activity (data not shown). The possible indirect effect of glucose through repression of GA synthesis [23] was also ruled out by treating the embryos with exogenous GA_3 (Fig. 5). The GA_3 treatment was unable to reverse the effects of glucose. A possible indirect effect of glucose mediated by an hypothetical glucose-triggered increase in ABA level was also ruled out. Indeed, the glucose treatment resulted in a decrease in the ABA content of the embryos (data not shown). Further analyses for the regulation of *RAmy1A* promoter function and comparison in promoter function between *RAmy1A* and *RAmy3D* genes will be needed to clarify the sugar repression of rice α-amylase gene expression.

Acknowledgements: We thank Dr. R.L. Rodriguez for providing us with the promoter fragment and cDNA clones of *RAmy1A* and *RAmy3D*. This research was supported in part by Grants-in-Aid no. 05276102 (to J.Y.) for Special Research on Priority Areas and no. 07660006 (to J.Y.) and 90096154 (to P.P.) from the Ministry of Education, Science and Culture (Monbusho), Japan. P.P. acknowledges a fellowship from the Japan Society for the Promotion of Science (1996–1997).

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