Analysis of Embryo-specific $\alpha$-Amylase Using Isolated Mature Rice Embryos

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Summary
As a first step to clarify the $\alpha$-amylase production in embryos, preparation of mass-isolated rice embryos was established. Morphological and biochemical characterization of the mass-isolated embryos revealed that the embryos are viable and suitable for characterization of $\alpha$-amylase production. Activity staining for $\alpha$-amylase revealed that the production of RAMy1A-encoded protein (isof orm A) is under hormonal control, but that is other isof orms including RAMy3D-encoded protein (isof orms G and H) are not. All isof orms were repressed by glucose. The physiological role of $\alpha$-amylase produced in the embryos was discussed.

Key Words: $\alpha$-amylase, gibberellin, rice (Oryza sativa), sugar repression, starch degradation, scutellar epithelium.

Introduction
Rapid germination and early seedling growth are critical for direct seedling cultivation and are important agronomic traits. Germination studies showed that $\alpha$-amylase (EC 3.2.1.1) activity is a primary factor and the expression of RAMy1A gene for $\alpha$-amylase is an important and possibly rate-limiting factor in determining seedling vigor in rice (Karrer et al., 1993). Yoon (1998) demonstrated the correlation between seedling emergence and amylolytic activity under low temperature and submerged soil conditions in 25 rice cultivars. A significant correlation of $\beta$-amylase activity with the emergence coefficients and seedling length was observed under the submerged soil condition at 18 °C, but that of $\alpha$-amylase was not. Further detailed studies on embryo-specific $\alpha$-amylase are needed to clarify which starch-degrading enzyme is critical for the direct seedling cultivation of rice. $\alpha$-amylase catalyzes the hydrolysis of $\alpha$-1,4 glucan bonds of starch in germinating cereals. Experimental evidence provided by in situ activity staining (Okamoto and Akazawa, 1979) and detection of mRNA (Ranjhan et al., 1992; Sugimoto et al., 1998) demonstrated that the biosynthesis of $\alpha$-amylase is initiated in the embryonic scutellar epithelium and then the biosynthesis occurs in aleu rone layer of the endosperm at later stages of germination.

In barley, isozymes of $\alpha$-amylase are classified into those having a low isoelectric point (pl) and a high pl, each group consisting of multiple isozymes (Khurshedd and Rogers, 1988). Genes for $\alpha$-amylase in rice form a family of ten genes located on five different chromosomes (Huang et al., 1992). These genes have been classified into three subfamilies, namely, RAMy1A, 1B and 1C; RAMy2A; RAMy3A, 3B, 3C, 3D, 3E and 3F as observed in several enzymes encoded by multigene families in cereals. In germinating wheat seeds, 27 isozymes have been identified (Gale et al., 1983). Ten $\alpha$-amylase isozymes (isof orm A, B, E, F, G, H, I, J, Y and Z) have been identified in suspension-cultured cells of rice having different pl values (Mitsu et al., 1996). From the results of amino acid sequencing of the purified isof orms, isof orms A and B, and isof orms G and H were identified as the gene products of RAMy1A and RAMy3D, respectively.

There are many reports that the biosynthesis of the enzyme is regulated by gibberellin (GA) and abscisic acid (ABA) at the transcriptional level (Jones and Jacobsen, 1991 for review). However, most of the studies were concerned with the gene transcription or biosynthesis of $\alpha$-amylase in the aleu rone layer cells. Studies on biosynthesis and its regulatory mechanisms of $\alpha$-amylase in the embryonic scutellar epithelium still remained controversial. Sugars affect GA synthesis (Radley, 1967) and $\alpha$-amylase production in barley scutellar tissue (Smith and Briggs, 1980). Beside phytohormones, recently barley $\alpha$-amylase genes (both high and low pl groups) have been reported to be controlled by sugars in the embryo, and not in the aleu rone, suggesting that $\alpha$-amylase activity is generally controlled by sugars in the embryo (Perata et al., 1997).

Differential expression between $\alpha$-amylase gene families in embryo and aleu rone of rice (Karrer et al., 1991) has been reported; that is, the expression of rice $\alpha$-amylase especially RAMy3D (also aAmy3) in the embryos is regulated by metabolizable sugars (Yu et al., 1991 and 1996; Karrer and Rodriguez, 1992). By in situ hybridization the sugar repression of rice $\alpha$-amylase gene RAMy3D has been shown to take place in scutellar epithelium cells in callus-forming rice embryos and that consensus sequences of G motif and TATCCA T/C motif of the promoter region are responsible for sugar repression (Mitsunaga et al., 1994; Umemura et al., 1998; Toyofuku et al., 1998). RAMy1A gene, which is clearly under hormonal control in the aleu rone (Itoh et al., 1995), is also under sugar regulation, although to a smaller extent when compared with that of RAMy3D (Morita et al., 1998). These results are consistent with those of isof orm analysis performed using suspension

**Abbreviations:** ABA, abscisic acid; GA, gibberellicacid; pl, isoelectric point.

Received March 16, 1998. Accepted September 18, 1998.
cultured cells of rice.

Herein to further characterize rice α-amylase isoforms, I examined the embryonic-specific α-amylase isoforms of rice and distinct profiles of expression of the isoforms (Ramy1A-encoded and Ramy3D-encoded proteins) in response to GA and metabolizable sugars using mass-isolated embryos of rice.

**Materials and Methods**

**Rice embryos**

Embryos of rice (Oryza sativa cultivar. Nipponbare) used in this study were prepared from fresh whole rice seeds by brief homogenization in a Waring blender. The ground material was passed through a graded wire mesh screen to remove endosperm tissues, and the sieved embryos were floated on a mixture of carbon tetrachloride and cyclohexane (25:10 v/v) (Yamaguchi et al., 1992). Embryos obtained were viable (more than 95%) as determined by ordinary seed germination count.

**Culture of isolated embryos and embryoless half-seeds of rice**

Twenty isolated embryos placed in the wells of a 24-well plate (no.3047; Falcon, New Jersey, U.S.A.) were surface-sterilized for 10 min with 1 ml of 0.25% solution of NaClO that contained 0.01% Tween 20 and then washed three times with 2.5 ml of sterile distilled water. The embryos were then incubated with 0.5 ml of culture medium (10 mM sodium acetate, pH 5.3, containing 2 mM CaCl₂) supplemented with or without GA₃, ABA, uniconazole or glucose, at 30°C in darkness.

To examine the induction of α-amylase by GA₃ in embryoless half-seeds, six half-seeds were placed in the wells of a 24-well plate. They were surface-sterilized for 10 min with 1 ml of a 1% NaClO solution that contained 0.01% Tween 20 and washed three times with 2.5 ml of sterile distilled water. They were then incubated with 0.5 ml of culture medium (10 mM sodium acetate, pH 5.3, containing 2 mM CaCl₂) supplemented with GA₃ at the designated concentrations at 30°C in darkness.

**Assay of α-amylase activity**

After a 4-day incubation, the embryos or embryoless half-seeds were homogenized with the culture medium plus an additional 0.5 ml of homogenizing buffer (100 mM Hepes·HCl, pH 7.6, containing 10 mM CaCl₂ and 0.1% Triton X-100), and then the homogenate was centrifuged at 18,500 xg for 10 min. The supernatant was used for measurements of enzymatic activity.

α-amylase activity was determined by the RBB-starch method of Hall et al. (1970). The sample (50 μl) and 450 μl of the suspension of substrate [2 g of RBB-starch (Amylolicatin Azure; Calbiochem, Los Angeles, CA) dissolved in 50 mM sodium acetate buffer that contained 10 mM CaCl₂] were incubated for 10 min at 25°C and the reaction was terminated by the addition of 200 μl of 18% acetic acid. After centrifugation at 18,500 xg for 5 min, the absorbance of the supernatant was measured at 595 nm. Units of activity were defined by the following equation: units = As / ACu; where As and ACu represent the absorbance at 595 nm of the reaction mixture and of a 1 M solution of CuSO₄, respectively (Mitsunaga and Yamaguchi, 1993).

**Zymogram of α-amylase**

Isoelectric focusing was performed with a Multiphor II Electrophoresis system (Pharmacia-LKB, Uppsala, Sweden) in accordance with the instructions from the manufacturer. Electrophoresis was performed on Ampholine gels (Ampholine PAG Plate; pH range 3.5–9.5; Pharmacia-LKB) at 1500 V for 1.5 h at 10°C. After isoelectric focusing, gels were incubated in a 1% solution of β-limit dextrin in 50 mM sodium acetate buffer that contained 10 mM CaCl₂ (pH 5.2) for 2 h. After soaking in distilled water, the gel was incubated in a solution of 1.3% I₂ and 3% KI for detection of amylase activity (Perata et al., 1992).

**Electron microscopy**

For electron microscopy, samples were fixed with 2% (w/v) glutaraldehyde for 2 h at room temperature, and then postfixed with 1% OsO₄ for 12 h at 4°C. They were dehydrated in a graded series of ethanol and propylene oxide, and embedded in Epon 812 resin. The thin sections were stained with uranyl acetate for 1 h and poststained with lead citrate for 5 min.

**Results and Discussion**

**Mass preparation of viable rice embryos**

Commercial rice embryos (germ) have been used for various biochemical and cell biological studies (for example, nuclei isolation by Yamaguchi et al., 1992). However, the commercial products are not viable, and the embryos isolated by hand have been used to clarify the mechanisms for α-amylase gene regulation (Karrer and Rodriguez, 1992; Perata et al., 1997). Hand picking, however, is tedious and time-consuming. Therefore, I isolated viable rice embryos in relatively large amounts according to the method of Johnston and Stern (1957). The rice embryos (Fig.1A) were prepared from chilled fresh seeds by brief homogenization in a blender. The ground material was then passed through a graded wire mesh screen and the sieved embryos were floated on an organic solvent. The embryos obtained were viable (more than 95%) as determined by ordinary seed germination count (Fig.1C).

**Morphology of mass-prepared rice embryos**

In rice, as in many other flowering plants, the embryo enters the quiescent stage during ripening of seeds. The cells of the quiescent (dry) embryo are poorly hydrated and lack mitotic activity (Fig.1A). Their metabolism is almost completely arrested. When the mass-prepared
embryos were imbibed, plumules (coleoptiles) appeared, but radicles did not (Fig.1B). The growth of rice seedling under anoxia is characterized by growth of coleoptile, without development of either the root or primary leaves (Perata et al. 1992). The results indicate that the growth conditions in this experiment are hypoxic or anoxic. The radicles also appeared from the mass-prepared embryos when they were placed on agar plate and grown under aerobic conditions (data not shown).

Fig.2 shows the morphological structure of scutellar epithelium cells in dry (A) and germinating (B) embryos. A unique feature of the epithelial cells at the early stage is an abundance of lipid-storing bodies (spherosomes) localized near the cell wall. There is a thick fibrous layer between the epithelium and the endosperm tissue, which has been reported by Okamoto and Akazawa (1979). In the mass-prepared embryos, the epithelium is covered with the fibrous layer to protect it from physical damage, indicating that the scutellar epithelium cells in the mass-prepared embryos are intact. This is important because the epithelium cells are the sole site of hydrolytic enzyme secretion in the embryonic tissues (Sugimoto et al., 1998). This experimental materials were considered suitable for further characterization of α-amylase isof orm in the embryos.

**Effect of gibberellin and sugars on α-amylase production in isolated embryos**

α-amylase is induced by GA in aleurone cells during germination of cereal seed. Here, the character of α-amylase produced by embryos was in comparison with that produced in embryoless half-seeds (aleurone cells) in rice. α-amylase activity in the mass-prepared embryos increased from day 1 up to day 4 even without GA₃ application (Fig.3), while no activity was observed in the embryoless half-seeds without GA₃ application. To confirm the GA-independence of the α-amylase production in the embryos, various amounts of GA₃ were applied and their effects on the enzyme production examined (Fig.4). GA₃ added to the culture medium at the concentration from 10⁻¹⁰ M to 3 × 10⁻⁵ M had no effect on the α-amylase production in the embryos, while the production of α-amylase in embryoless half-seeds was strikingly promoted by GA₃ at around 10⁻⁸ M, indicating that a saturating amount of GA to induce α-amylase production might be synthesized in the embryo even in the absence of exogenous GA₃ (Perata et al., 1997).

To examine the dependency of embryo-specific α-amylase production further, the effects of ABA as an antagonist of the action of GA, and uniconazole-P, as
Fig. 3. Effect of exogenous GA$_3$ on $\alpha$-amylose production in embryos and embryoless half-seeds.
Isolated embryos (●, ○) and embryoless half-seeds (■, □) of rice were incubated with culture medium and allowed to germinate for up to 4 days. The culture medium was supplemented with (●, ■) or without (○, □) 1 $\mu$M GA$_3$. $\alpha$-Amylose activity measured by RBB-starch method (Hall et al., 1970) is expressed as activity unit per 10 samples (for the embryos) and per one sample (for the embryoless half-seeds).

Fig. 4. Does response curve of GA$_3$-induced $\alpha$-amylose production in embryos and embryoless half-seeds.
After a 4-day incubation with a various concentration of GA$_3$, embryos (●) and embryoless half-seeds (■) were extracted and the $\alpha$-amylose activity was measured. $\alpha$-Amylose activity measured by RBB-starch method (Hall et al., 1970) is expressed as activity unit per 10 samples (for the embryos) and per one sample (for the embryoless half-seeds).

Fig. 5. Effects of ABA, uniconazole-P and sugars on production of $\alpha$-amylose isoforms fractionated by isoelectric focusing.
Embryos of rice were incubated for 4 days with the following chemicals. Panel A: control (lane 1), 1 $\mu$M GA$_3$ (lane 2), 1 $\mu$M ABA (lane 3), 10 $\mu$M ABA (lane 4), 0.1 $\mu$M uniconazole-P (lane 5) and 10 $\mu$M uniconazole-P (lane 6). Panel B: control (lane 1), 1 $\mu$M GA$_3$ (lane 2), 90mM glucose (lanes 3), 1 $\mu$M GA$_3$ + 90mM glucose (lanes 4). Samples (30 $\mu$l) after protein extraction (see Assay of $\alpha$-amylose activity, Materials and Methods) were applied on Ampholine gel. Activity staining of $\alpha$-amylose was described by the method of Perata et al. (1992).

A was repressed by ABA and uniconazole treatments, while other isoforms were not (lanes 3–6). The effects of glucose on the production of $\alpha$-amylose isoforms were also examined (Fig. 5B). All the isoforms were repressed by 90 mM glucose (lanes 3, 4 in Fig. 5B), indicating that glucose is a strong repressors of the $\alpha$-amylose production in the embryos. These results clearly demonstrated by the isofom analyses using mass-isolated embryos that production of both Ramyla and 3D-encoded proteins is under sugar control, while that of Ramyla-protein is also under hormonal control. These results are consistent with the previous report using callus-forming rice embryos that Ramyla gene is under hormonal control, but Ramyl3D gene is not (Morita et al., 1998).

$\alpha$-amylose produced in cereal embryos
$\alpha$-amylose play a major role during the degradation of native starch granules (Beck and Ziegler 1989; Perata et al., 1992), although only through the concerted action of $\alpha$-amylose, $\beta$-amylose, debranching enzyme, and $\alpha$-glucosidase, starch is hydrolyzed completely (Sun and Henson, 1991; Guggielmi et al., 1995). Isoform A, Ramyla-encoded protein, definitely functions in vivo
in the degradation of starch during germination of seeds. What is the physiological role of RAMy3D-encoded protein, isoforms G and H in cereal seed germination? There is increasing evidence showing that RAMy3D gene expression is controlled by feed-back metabolite (carbohydrate) repression. In fact, analysis of the α-amylase isoforms showed that the production of the RAMy3D-encoded protein is repressed by the presence of glucose in rice embryos (Fig.5B). Imbition of the embryos may trigger the production and secretion of RAMy3D-encoded α-amylase to degrade endosperm starch close to the embryo and the subsequent absorption of degraded carbohydrates through scutellar epithelium cells may result in activation of quiescent embryo, although the gene transcription would be finally repressed by the degradation products of starch. The production of RAMy3D-encoded α-amylase in the embryos is probably a critical step for this process.

In contrast to the well-known expression pattern during germination, it is almost unknown how α-amylase expression is regulated during seed development. In situ hybridization revealed that the RAMy1A gene is expressed in the scutellar epithelium during early maturation stage, but not at the late stage (Sugimoto et al., 1997). Interestingly, RAMy1A transcripts were not detectable in the aleurone layer throughout the seed development. Hoecker et al. (1995) have reported that Viviparous-1 (VP1), a transcriptional activator of maize, inhibited the induction of α-amylase genes in aleurone cells of the developing maize seeds. It is likely that VP1 protein and endogenous ABA play a crucial role in the repression of α-amylase gene expression in developing seeds of rice. Repression of α-amylase production in developing seeds seems to be critical to prevent pre-harvest sprouting in cereals.

I showed in this report that the mass-isolated rice embryos are suitable system to clarify α-amylase production in the embryos. α-Amylese production is a crucial step for degradation of endosperm starch and might be one of the rate-limiting factors in determining seedling vigor in rice. Utilization of starch degradation products in the embryos, that might be also an important factor for seedling process, would need apoplastic transport of sugars across membrane of scutellar epithelium cells. Indeed termination of α-amylase production in the scutellar epithelium may result in functional changes from the initial secretion of starch degrading enzymes to the absorption of degraded products from endosperm. The mass-isolated embryo system will be also useful to examine this speculation.

Acknowledgments

Thanks are due to Dr. T. Akazawa and K. Aratani for their support and stimulating discussion, and also to Dr. K. Kojima for his help with the EM of rice embryos. This research was supported by no. 07660006 from the Ministry of Education, Science and Culture (Mon-busho), Japan. This research was also supported in part by Iijima Memorial Food Science Foundation.

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