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Endogenous Gibberellin A_1 Level and α-Amylase Activity in Germinating Rice Seeds

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Abstract. The role of endogenous gibberellin A_1 (GA_1) in the induction of α-amylase activity was investigated during germination of rice (*Oryza sativa* L.) seeds. The level of endogenous GA_1 and the α-amylase activity in the seeds of normal rice, cv. Nipponbare, increased simultaneously from 3 days after the imbibition of water. The α-amylase activities in the dwarf rice, cv. Waito-C and Tan-ginbozu, were less than that in the normal rice. The level of endogenous GA_1 and α-amylase activity were decreased in proportion to the concentration of a growth retardant, uniconazole. The retardation in α-amylase activity caused by the treatment of uniconazole was recovered by the application of exogenous GA_1. These results indicate that the endogenous GA_1 biosynthesized de novo regulates α-amylase production in germinating rice seeds.

Key Words. α-Amylase—Germination—Gibberellin—*Oryza sativa* L.

Exogenous application of gibberellins (GAs) can induce α-amylase in the aleurone layer of the embryoless seeds of barley (*Hordeum vulgare* L.) (Jacobsen et al. 1995). Endogenous GA levels in germinating barley seeds were analyzed to reveal that GA_1 was the major endogenous GA and that the increase in the level of GA_1 preceded the induction of α-amylase activity (Kobayashi et al. 1995, Yamada 1982). These results indicate that the GA_1 biosynthesized de novo in the embryo diffuses to the aleurone layer to induce α-amylase during germination of barley seeds.

In rice (*Oryza sativa* L.) seeds, GAs could induce α-amylase activity (Tanaka et al. 1970), and those GAs that have a 3β-hydroxyl group were supposed to be active per se for the induction of α-amylase (Soh et al. 1994). Mitsunaga and Yamaguchi (1993) reported that α-amylase activity was less in the GA-deficient dwarf rice, cv. Tan-ginbozu (*dx* mutant), Waito-C (*dy* mutant), and Kotake-tamanishiki, than in the normal rice, cv. Nipponbare. They also reported that uniconazole, an inhibitor of GA biosynthesis, prevent the induction of α-amylase in rice seeds. The α-amylase activity in the seeds treated with uniconazole was rescued by an application of GA_3. These results suggest that endogenous GA is essential for the induction of α-amylase activity in rice seeds.

We have investigated the endogenous GAs in rice and found that 13-hydroxylated GAs are dominant in the vegetative tissues (Takahashi and Kobayashi 1989). In a previous paper we reported the occurrence of GA_1, GA_{19}, GA_{20}, and GA_{53} in germinating rice seeds (Choi et al. 1995a). GA_1 was the only 3β-hydroxylated GA that was detected in the seeds. Based on this result, it was suggested that GA_1 was responsible for the induction of α-amylase in germinating rice seeds.

Although all of these results indicate the involvement of endogenous GA_1 in the induction of α-amylase in germinating rice seeds, it is necessary to investigate the correlation between the endogenous GA_1 level and α-amylase activities to confirm the importance of GA_1 in α-amylase induction.

In this paper we report the development of the levels of GA_1, abscisic acid (ABA), and α-amylase activity during germination of rice seeds. ABA was examined because of its inhibitory effect in the induction of α-amylase and its potential involvement in the regulation of the induction of α-amylase during germination (Fincher

Abbreviations: GA(s), gibberellin(s); ABA, abscisic acid; AE fraction, acidic ethyl acetate-soluble fraction; HPLC, high performance liquid chromatography; R_t, retention time, GC-SIM, gas chromatography-selected ion monitoring.

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1989, Lenton and Appleford 1990). The level of GA_1 and α-amylase activity in dwarf rice seeds and the seeds treated with uniconazole during germination are also discussed.

Materials and Methods

Plants

Rice (*O. sativa* L. cv. Nipponbare, Tan-ginbozu, and Waito-C) seeds were hulled and surface sterilized in 1% solution of NaOCl for 30 min followed by an imbibition in 10 mM HCl for 10 min and were then washed with sterile distilled water. Ten seeds were soaked in a 15-mL sterile tube filled with 2 mL of 10 mM sodium acetate buffer pH 5.3, containing 10 mM $CaCl_2$ with or without uniconazole, and they were cultured with reciprocal shaking (100 rpm) in a dark room at 28°C for 7 days. When necessary, GA_1 dissolved in 70% ethanol (9 μL) was added to the solution 3 days after the beginning of incubation. Germinating seeds were harvested every day for the measurement of α-amylase activity and for analysis of endogenous GA_1 and ABA. The harvested tissues were measured for shoot length.

Chemicals

Soluble potato starch was purchased from Merck Co. Uniconazole [(*E*)-1-(4-chlorophenyl)-4,4-dimethyl-2-(1,2,4-triazol-1-yl)-1-penten-3-ol (S-3307 D)] was a gift from Dr. Izumi, Sumitomo Chemical Co.

Assay of α-Amylase Activity

The harvested tissues were homogenized in an ice water bath with the cultured broth and centrifuged at 15,000 rpm for 10 min at 4°C. To inactivate β-amylase activity, the supernatants were heated to 60°C for 30 min and centrifuged again according to the method of Grosselindemann et al. (1991). The supernatants were assayed for α-amylase activity. Determination of α-amylase activity was done according to the following procedure. The starch suspension (2 g of potato starch in 100 mL of distilled water) was boiled for 1 min, cooled, and centrifuged at 10,000 rpm for 10 min. The clear supernatant that separated from the unsolubilized starch in the bottom of the tube was used for the assay. An aliquot of enzyme solution was added to a 1.5-mL microcentrifuge tube with 0.2 mL of starch solution, 0.2 mL of 0.1 M sodium acetate buffer, pH 5.3, and 0.2 mL of 10 mM $CaCl_2$. The total volume of the solution was adjusted to 1.2 mL with distilled water, and the solution was kept at 37°C for 10 min. The reaction was stopped by adding 0.4 mL of KI/I_2 solution (0.5% KI, 0.05% I_2, and 0.05 N HCl). The absorbance at 620 nm was measured, and 1 unit of the α-amylase activity was defined as decrease of 0.1 O.D. in A_{620}. The absorbance of control (without the addition of enzyme solution) was 1.0–1.3.

Extraction and Purification of GA_1 and ABA

The harvested tissues in 80% methanol (10 mL/g, fresh weight) were homogenized by a blender and kept at −20°C (overnight). After filtration, the residue was immersed in 80% methanol (10–20 mL/g, fresh weight) for 1 h and filtered again. The filtrates were combined, and the extracts equivalent to 4–8 g, fresh weight, were concentrated after the addition of 5 ng of [1,2,2,3,6,-$^2H_5$]GA_1 and 100 ng of [6-$^2H_3$]ABA as internal standards. The aqueous residue was then solvent fractionated to give an acidic ethyl acetate-soluble (AE) fraction (Yokota et al. 1980). The AE fraction was prepurified with a Bond Elut C_{18} column (Varian, Harbor City, CA) and submitted to a purification by three steps of HPLC on the columns of Hitachi gel 3011, Shodex GPC HF-2001, and Nucleosil 5 C_{18} (Choi et al. 1995b). At the final step of HPLC purification on the ODS column, the fractions corresponding to GA_1 (R_t 8–11 min) and ABA (R_t 13–16 min) were collected and concentrated.

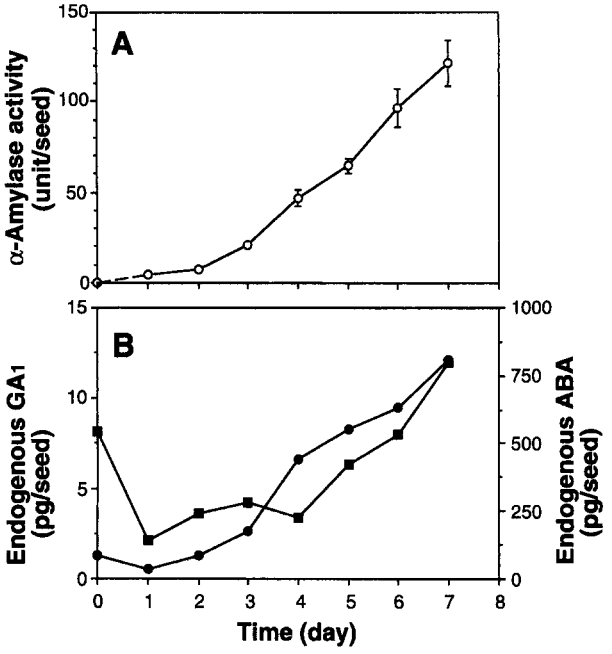

Fig. 1. α-Amylase activity (**A**) and the levels of endogenous hormones (**B**), GA_1 (●) and ABA (■), in germinating rice seeds (cv. Nipponbare). Seeds were hulled and incubated with 2 mL of 10 mM sodium acetate buffer containing 10 mM $CaCl_2$. *Vertical bars* in α-amylase activity represent standard errors of the mean of three replicates.

GC-SIM Analysis

The quantitative analysis of endogenous GA_1 and ABA was performed by GC-SIM with the procedure described previously (Kobayashi et al. 1995).

Results

Levels of GA_1, ABA, and α-Amylase Activity during the Seed Germination of Normal Rice

The rice (cv. Nipponbare) seeds were cultured in test tubes for 7 days, and they were harvested every day for the analysis. As shown in Fig. 1, α-amylase activity was detectable 1 day after the imbibition with water (day 1). The activity increased at a constant rate from day 3 until day 7. The levels of endogenous GA_1 and ABA were quantified by GC-SIM analysis using deuterium-labeled

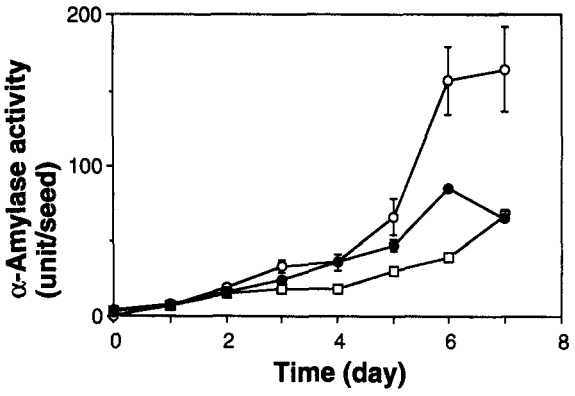

Fig. 2. α-Amylase activity in Nipponbare (○), Waito-C (●), and Tan-ginbozu (□). Each point shows the average of data from ten rice seeds, and *vertical bars* represent standard errors of the mean of three replicates.

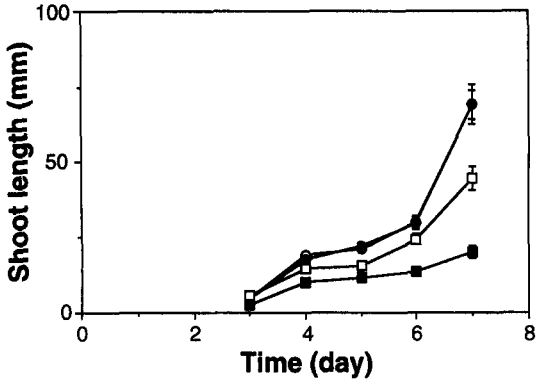

Fig. 3. Effects of uniconazole on the length of shoot in germinating rice seeds (cv. Nipponbare). Hulled seeds were incubated with 0 nM (○), 1 nM (●), 10 nM (□), and 100 nM (■) uniconazole. *Vertical bars* represent the standard errors of the mean of three replicates.

internal standards. Once the level of endogenous GA_1 decreased at day 1 and then increased from day 2. Its level increased significantly after day 3, at the stage that shoots started elongation. The level of ABA declined rapidly at day 1 and increased gradually after that.

Development of α-Amylase Activity in Dwarf Rice

Endogenous GA_1 levels in the shoots of dwarf mutants of rice, cv. Tan-ginbozu and Waito-C, were lower than that in the normal (tall) cultivar, cv. Nipponbare (Kobayashi et al. 1989). The level of endogenous GA_1 in the young leaf tissues of the dwarf rice was approximately 10% of that in the normal rice (Choi et al. 1995b). We investigated the development of α-amylase activity in these dwarf rice. As shown in Fig. 2, the α-amylase activities in the dwarf rice were similar to that in Nipponbare until day 2, whereas the increase in activity was much less in the dwarf rice after day 4. Waito-C showed relatively higher α-amylase activity than Tan-ginbozu. This is consistent with the previous result that the shoots of Waito-C had a higher GA_1 level than those of Tan-ginbozu (Kobayashi et al. 1989).

Effect of Uniconazole in the Induction of α-Amylase Activity and GA_1 Level

Uniconazole is a plant growth retardant that inhibits the early stage of GA biosynthesis, namely the oxidation of *ent*-kaurene to *ent*-kaurenoic acid (Izumi et al. 1985). Mitsunaga and Yamaguchi (1993) reported that uniconazole inhibits the induction of α-amylase in rice seeds. However, in their study even 100 μM uniconazole could not give more than a 50% reduction in the α-amylase activity in the normal rice (Nipponbare). We reexamined the effect of uniconazole on the induction of α-amylase activity in conjunction with the level of endogenous GA_1. Seeds of Nipponbare were incubated with uniconazole (0, 1, 10, and 100 nM), and the germinating seeds were harvested every day from day 3 until day 7 for a measurement of α-amylase activity. The shoot length was measured at the time harvested, and the samples at day 5 were subjected to GA_1 analysis. As shown in Figs. 3 and 4, the shoot length decreased in proportion to the concentration of uniconazole, as was the case for the α-amylase activity. When compared at day 5, the α-amylase activities in the seeds treated with 1, 10, and 100 nM uniconazole were 94, 35, and 17% of that in the untreated seeds, respectively. The levels of endogenous GA_1 in the seeds treated with 10 and 100 nM uniconazole at day 5 were reduced to 35 and 12% of that of untreated seeds (6.5 pg/seed), respectively, whereas there was no decrease in the GA_1 level after the treatment with 1 nM with uniconazole.

In our experiments 100 nM uniconazole almost completely suppressed the increase in α-amylase activity in the seeds of Nipponbare, which was inconsistent with the results by Mitsunaga and Yamaguchi (1993). This inconsistency may be caused by the different growth conditions; for example, rice seeds were grown in a liquid culture in the present study but in agar culture in the previous one.

Effect of GA_1 Application to the α-Amylase Activity in the Seeds Treated with Uniconazole

GA_1 was applied to the seeds treated with uniconazole, and the α-amylase activity in the seeds was measured to confirm that the reduction of the α-amylase activity was due to the deficiency of endogenous GA. Seeds of Nipponbare were incubated with 100 nM uniconazole for 3

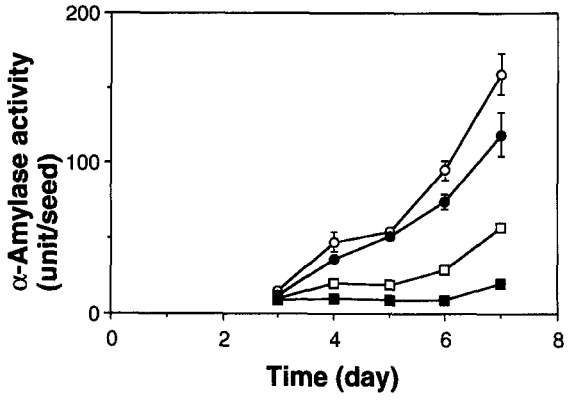

Fig. 4. Effects of uniconazole on α-amylase activity in germinating rice seeds (cv. Nipponbare). For symbols, see the legend of Fig. 3.

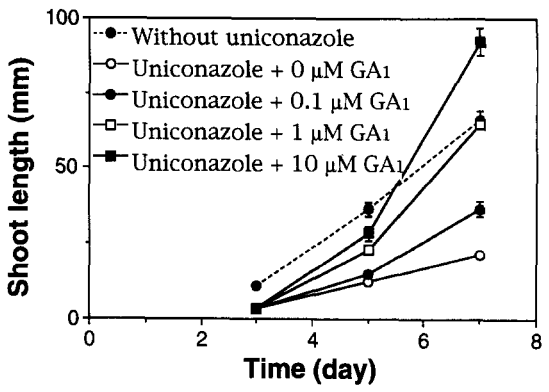

Fig. 5. Effects of GA_1 on the length of shoot treated with 100 nM uniconazole. Hulled seeds were incubated with 100 nM uniconazole, and GA_1 was applied at day 3. Plants were harvested at day 7 for measurements. *Vertical bars* represent the standard errors of the mean of three replicates.

days, and GA_1 was applied to the culture at day 3 to give concentrations of 0.1, 1, and 10 μM. The α-amylase activity was analyzed from day 3 until day 7 (Fig. 5). As shown in Fig. 6, the α-amylase activity was almost recovered by the treatment of 0.1 μM GA_1, the lowest concentration investigated, but the shoot length was not completely recovered at this concentration (Fig. 5). This result indicates that induction of α-amylase activity requires a lower amount of GA_1 than shoot elongation in the germinating rice seeds.

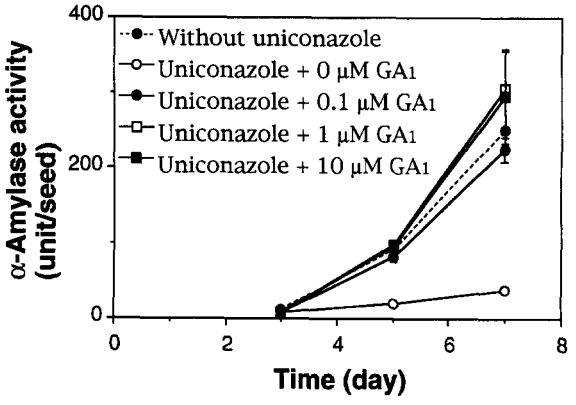

Fig. 6. Effects of GA_1 on α-amylase activity. For analytical conditions, see the legend of Fig. 5.

Discussion

The previous study revealed that GA_1 was the major active GA in the germinating rice seeds (Choi et al. 1995a). The following results in the present study confirm the role of endogenous GA_1 on the induction of α-amylase in germinating rice seeds. (1) A simultaneous increase was observed in the GA_1 level and α-amylase activity during germination (Fig. 1). (2) α-Amylase activities in the dwarf rice were lower than that in the normal rice (Fig. 2), and the activity was also depressed in the seeds treated with uniconazole (Fig. 4), which reduced the GA_1 level in the seeds. (3) The α-amylase activity in the seeds treated with uniconazole was recovered by the application of GA_1 (Fig. 6).

α-Amylases in rice are encoded by a family of genes which can be divided into three subfamilies, *RAmy1*, *RAmy2*, and *RAmy3* (Huang et al. 1990a, 1990b). The expression of *RAmy1A* could be induced by the exogenous application of GA_3 (O'Neill et al. 1990), whereas expression of *RAmy3D* was regulated by a variety of sugars (Karrer and Rodriguez 1992). Okamoto and Akazawa (1979) reported that the α-amylase activity was first detectable in the epithelium septum 12 h after seed imbibition. The activity in the aleurone layers was detected 2 days after seed imbibition. Ranjhan et al. (1992) showed that mRNA of α-amylase detected in the scutella epithelium was primarily due to the expression of the *RAmy3D* gene and that in the aleurone layers, the *RAmy1A* gene. These previously reported findings and our present results suggest that the endogenous GA_1 synthesized de novo induces *RAmy1A* gene expression in the aleurone layers, whereas expression of *RAmy3D* in the scutella epithelium at the initial stage of germination may be independent of the regulation by GA_1. Indeed, α-amylase activities in the dwarf rice seeds were similar to that of normal rice until day 2, and even the highest concentration of uniconazole did not nullify the α-amylase activity (Fig. 4). Further investigation at the molecular level will give an answer to this subject.

It is known that ABA suppresses the expression of genes induced by GA during germination (Fincher 1989). In our results the level of endogenous ABA in germinating rice seeds decreased rapidly at day 1 and

remained at a relatively lower level until day 4, suggesting that endogenous ABA also concerns with the regulation of α-amylase induction in the aleurone layer. A similar development pattern for ABA was observed in germinating barley seeds (Kobayashi et al. 1995). However, in barley seeds, the maximum GA_1 level was observed at day 2 (Kobayashi et al. 1995), and the α-amylase activity fully increased within 48 h after the start of imbibition with water (Grosselindemann et al. 1991). In rice seeds, the α-amylase level started increasing at day 3, although the ABA level was already decreased at day 1 (Fig. 1). Unlike GA_1 levels, the development of ABA levels did not correlate directly to the induction of α-amylase activity in these cereal grains.

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