Pergamon

0031-9422(95)00380-O

Phytochemistry, Vol. 40, No. 6, pp. 1613–1616, 1995

0031-9422/95 $9.50 + 0.00

ACTIVITY OF STARCH SYNTHASE AND THE AMYLOSE CONTENT IN RICE ENDOSPERM

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(*Received in revised form* 29 *March* 1995)

Key Word Index—*Oryza sativa*; Gramineae; rice; endosperm; starch synthesis; starch synthase; amylose; low temperature.

Abstract—The content of amylose in endosperm of non-waxy japonica rice (*Oryza sativa* cv Akitakomachi) was increased by lowering the growth temperature from 25° to 15° during the ripening period. The activities of sucrose synthase, ADPglucose pyrophosphorylase, starch branching enzyme (Q-enzyme) and soluble starch synthase in endosperm developed at 15° were lower than or similar to those at 25°, when compared on a endosperm basis at the similar ripening stage. In contrast, the activity of starch granule-bound starch synthase, which is considered to be indispensable for amylose synthesis, was higher by 3–3.5-fold in the endosperm developed at the low temperature than that at the high ambient temperature. The results suggest that the low temperature specifically accelerates the expression of the bound starch synthase gene (waxy gene) in rice endosperm, which resulted in elevated amylose biosynthesis in the endosperm when developed at lower temperatures.

INTRODUCTION

Starch of non-waxy rice endosperm consists of two components: amylose and amylopectin. The amylose to amylopectin ratio has been considered to affect the quality of rice [1, 2]. It was reported that the amylose content per endosperm of rice positively correlated with the level of Wx protein (bound starch synthase) in endosperm [3]. Sano [4] has revealed that there are two types of Wx alleles—Wxa and Wxb—in rice. A lower temperature during the ripening period increases the amylose content in endosperm of typical japonica rice which possesses Wxb, while no distinct trend is seen in the indica type or in the isogenic line of japonica type with Wxa [1–3, 5, 6]. Sano *et al.* [6] also reported that a lower temperature during the ripening period increased the amount of Wx protein in japonica rice. Moreover, Northern-blot analysis indicated that the waxy gene expression was regulated by temperatures at the transcriptional level [7]. The steady-state level of the transcript increased by exposing the plants to a lower temperature of 18°.

There are extensive works in relation to temperature and enzymes involved in starch synthesis in endosperm of wheat [8–11]. Soluble starch synthase is considered to be the key enzyme under the heat stress condition since the activity of the enzyme specifically declined above 25°. Some information is available as regards rice [8], which also deal with heat stress on the endosperm.

Though the effect of growth temperature on expression of *Wxb* gene, synthesis of Wx protein (bound starch synthase) and content of amylose in rice endosperm have been studied as described above, its effect on the activity of bound starch synthase has not yet been revealed. In addition, it must be important to investigate activities of other enzymes associated with amylose and/or amylopectin synthesis in rice endosperm since the amylose to amylopectin ratio has been affected. In this paper, developing patterns of major enzymes in starch synthesis; sucrose synthase, ADPglucose pyrophosphorylase (ADPGlc PPase), starch branching enzyme (Q-enzyme), soluble starch synthase and bound starch synthase, and amylose levels in endosperm of non-waxy japonica rice (*Oryza sativa* cv Akitakomachi) developed under 15° (low temperature) and 25° (medium temperature) were examined.

RESULTS AND DISCUSSION

A shown in Fig. 1, both fresh and dry weight of hulled rice grain developed at 25° increased greatly up to 17 days after flowering (DAF). Lowering the temperature to 15° elongated the grain-filling duration from 31 to 48 days. At 11 DAF, the dry weight of hulled rice reached 52% that of the mature sample at 25°, whereas it reached only 7% at 15°. Because of the difference in grain-filling

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rate under different temperatures, the early milky stage, the mid-milky stage and the dough stage which were observed at 8, 11 and 14, DAF respectively, at 25° correspond to 17, 22 and 27 DAF, respectively, at 15°. Nevertheless, at maturity (31 DAF at 25°, 48 DAF at 15°, respectively), the ratios of fresh and dry weight of hulled rice developed at 15–25° were 98 and 96%, respectively. The optimal temperature for grain-filling rate has been studied in rice [12–16] or in wheat [16, 17]. It was shown that in japonica rice with the increase in ambient temperature, grain-filling rate became faster up to about 30°. On the other hand, the temperatures in the range from 16° to 25° did not affect the final weight of grain [12, 13, 15, 16]. Therefore, the present results are basically consistent with the previous reports.

Starch content per hulled rice at maturity was 6% lower at 15° than that of 25° (Table 1). The difference in starch content, however, became negligible when they were compared on a dry weight basis to remove the difference in dry weight of hulled grain between the temperature treatments. In contrast, lowering the temperature increased the amylose content in hulled rice by 28% (Table 1). It should be noted that the differences in quantity of dry matter or starch in hulled grain were not so large, while amylose level, which is considered to be one of the major factors in determining rice quality, was greatly affected by the temperature treatment, in agreement with the results reported [1–3, 5].

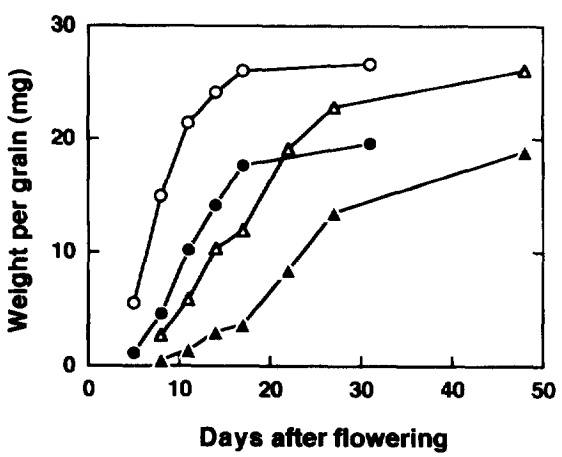

Fig. 1. Time courses of fresh weight and dry weight of grain (hulled rice) developed under different ambient temperatures after flowering. Values are mean of at least 10 grains. (○) 25° fresh wt; (●) 25° dry wt; (△) 15° fresh wt; (▲) 15° dry wt.

The activities of bound starch synthase is widely considered to be indispensable to generate amylose. However, it cannot exclude the possibility that not only the activity of bound starch synthase but also the balances of activity of enzymes involved in starch synthesis in rice endosperm are important to determine the ratio of amylose to amylopectin. Therefore, we examined changes in activities of bound starch synthase and other major enzymes involved in starch synthesis in endosperm during the course of its development at 25° and 15°. Figures 2 and 3 show that at 25° activities per endosperm of sucrose synthase, ADPGlc PPase and soluble starch synthase represented the peak values as 11 DAF, while those values were extremely low at 15° then. When compared at the mid-milky stage (11 DAF at 25° and 22 DAF at 15°), the activities of sucrose synthase, ADPGlc PPase, Q-enzyme and soluble starch synthase at 15° were in the range of 69–102% of the activities at 25°. In contrast, the activity of bound starch synthase in endosperm grown at 15° was 331% of that at 25°.

Amylose content in rice endosperm was reported to be determined by the ambient temperature at an early development stage (from 5 to 15 days after anthesis under 25°) [5]. As for bound starch synthase activity, Villareal and Juliano [18] presumed that the ambient temperature may affect the activity of bound starch synthase in the developing rice grain, although they did not show any data. The present data suggest that the selectively higher activity of bound starch synthase induced by the low temperature causes the higher amylose content in the hulled rice. On the other hand, in wheat neither the activity of insoluble starch synthase (or bound starch synthase) in endosperm [9] nor the amylose content in kernel [19] was affected greatly by temperature treatment.

The fine structure of amylopectin in rice endosperm was reported to be affected by the temperature during ripening [20]. Higher environmental temperature increased the amount of long B chains in amylopectin and decreased that of short B chains. As shown in Figs 2 and 3, the ratio of activity of soluble starch synthase at 15–25°

Table 1. Effects of temperature on dry weight and contents of starch, amylose and amylopectin (per mg hulled rice) in hulled rice grain at maturity

Temperature (°)	Dry wt	Starch	Amylose	Amylopectin
25	19.6	15.1 ± 0.9	2.27 ± 0.05 (15.0%)	12.84 ± 0.06 (85.0%)
15	18.9	14.2 ± 0.2	2.91 ± 0.04 (20.5%)	11.29 ± 0.04 (79.5%)

The data for dry wt are from Fig. 1. The data for starch, amylose and amylopectin represent mean ± s.e. of three replicate measurements. The data in parentheses represent amylose or amylopectin percentage in total starch. For measurements of starch, amylose and amylopectin embryos were removed from hulled rice.

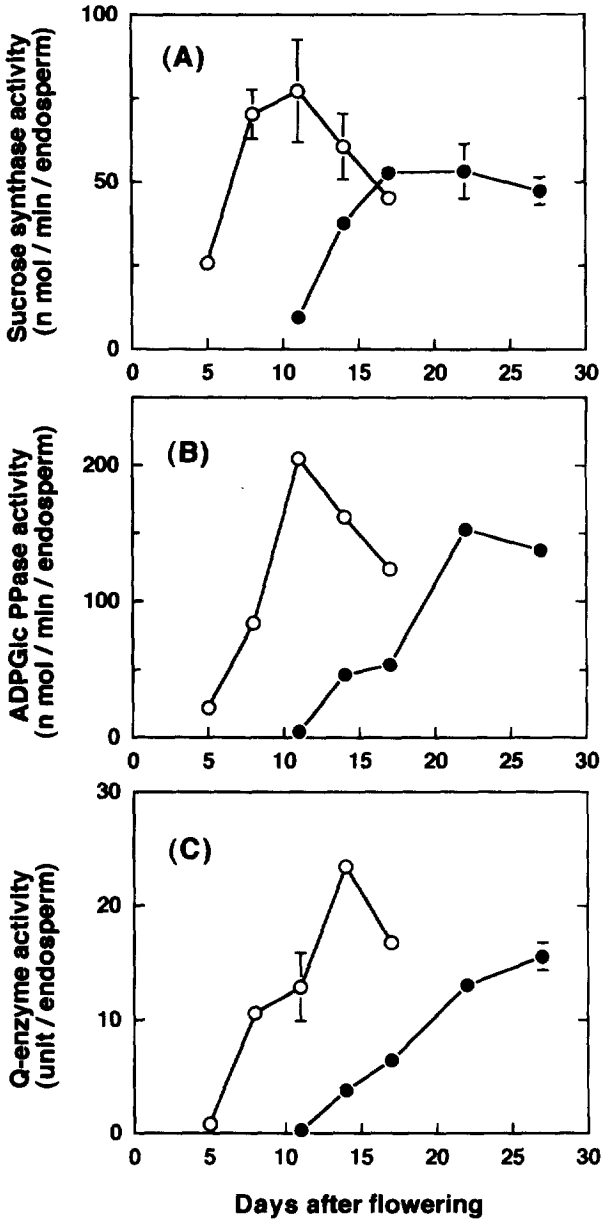

Fig. 2. Comparison of activity of sucrose synthase (A), ADPGlc PPase (B) and Q-enzyme (C) in rice endosperm developed under 25° and 15° after flowering. The mean ± s.d. is shown ($n = 4$). The absence of error bars indicates that the size of error does not exceed the size of the symbol. (○) 25°; (●) 15°.

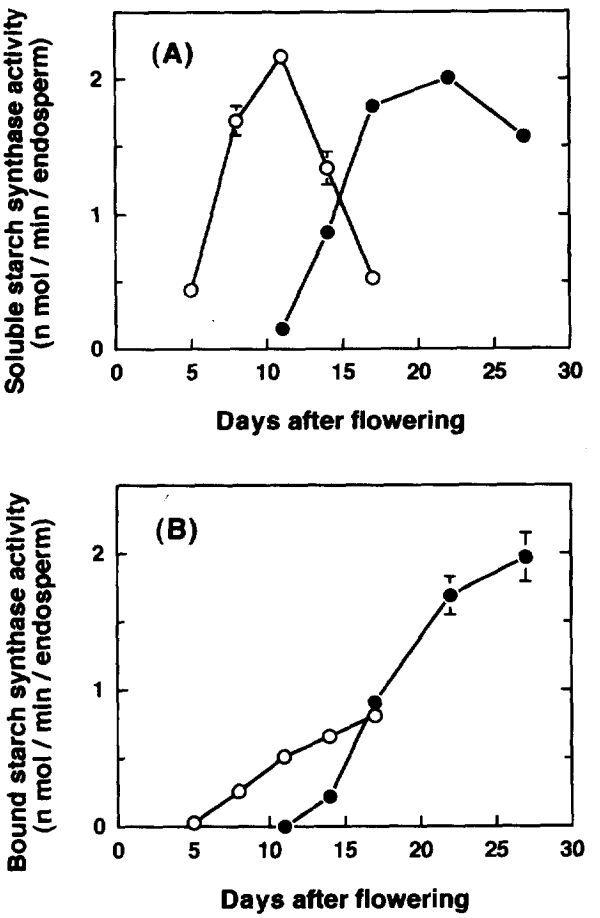

Fig. 3. Comparison of activity of soluble starch synthase (A) and bound starch synthase (B) in rice endosperm developed under 25° and 15° after flowering. The mean ± s.d. is shown ($n = 3$). The absence of error bars indicates that the size of error does not exceed the size of the symbol. (○) 25°; (●) 15°.

at the maximum value seemed to be higher (*ca* 93%) than those (65–75%) of sucrose synthase, ADPGlc PPase and Q-enzyme. Soluble starch synthase elongates α-1,4 bond in amylopectin.

EXPERIMENTAL

Plant material. Twenty seeds of japonica rice (*Oryza sativa* cv Akitakomachi) were sown in a circle in plastic pots, and grown in a greenhouse under natural daylight until the flowering. Tillers were cut once or twice a week during the vegetative stage. The grains located directly on the second to fourth upper primary branches (except the two grains from apex) were used in this study. The grains were marked on the hull at the day of flowering. One day after flowering, plant materials were moved into growth chambers in which the temp. was maintained at 15 or 25°. The grains were harvested several times during the maturing. Samples were stored at −85° until used. Grains were hulled and fr. wt was determined from the mean of at least 10 hulled rice. Dry wt of hulled rice was measured after drying the samples used for fr. wt measurement at 105° until it becomes constant.

Measurement of starch. To measure the starch content, *ca* 2 g of hulled rice with embryo removed was crushed with a grain crusher. 20 ml of DMSO and 5 ml of 8 M HCl were added to 100 mg of the rice powder, and incubated at 60° for 30 min. The soln was filled up to 100 ml with H_2O after the pH had been adjusted to 4–5 with 5 M NaOH. Then 300 μl of the resultant soln was incubated with 100 μl of 100 mM NaOAc buffer (pH 4.8) containing amyloglucosidase (3 U; Seikagaku Co., Tokyo, Japan) for 1 hr at 55°. The reaction was terminated

by heating the mixture at 100° for 1 min. The soln was transferred into an Eppendorf tube and centrifuged at 15000 rpm for 10 min. A portion (300 μl) of the supernatant was taken and mixed with 200 μl of 150 mM HEPES–NaOH buffer (pH 7.4) containing 10 mM $MgSO_4$, 3.2 mM NADP and 10.8 mM ATP. The starch content was assayed by measuring the increase in *A* at 340 nm after the addition of 1 μl each of hexokinase (1.4 unit) and glucose-6-phosphate dehydrogenase (0.35 unit), respectively.

Measurement of amylose. Starch prepn was performed according to Refs [5, 21], except we used hulled rice which removed the embryo, instead of milled rice. Debranching of starch by isoamylase and fractionation of the debranched starch on Sephadex G-75 column were performed by a modification of the method reported in Ref. [22]. The starch prepns (*ca* 40 mg) were gelatinized by 1 ml of 1 M NaOH over night at 5°. The gelatinized starches were neutralized with 1 M HCl and were debranched by crystalline *Pseudomonas* isoamylase (1475 unit; Hayashibara Biochemical, Inc., Okayama, Japan) in 5 ml of 830 mM NaOAc buffer, pH 3.5, at 40° for 24 hr. The debranched starches were dried *in vacuo* at 40°, and dissolved with 1 ml of 1 M NaOH. The sample soln added to 1 ml of H_2O was applied to a column of Sephadex G-75 (22 mm dia. × 95 cm length) that had been equilibrated with 0.2% NaCl/0.02 M NaOH. The sample was eluted with the same soln at a flow rate of 0.25 ml min^{-1}. Frs were collected at 5 ml intervals and neutralized with 1 M HCl. The carbohydrate content in each fr. was measured by the phenol–H_2SO_4 method. Amylose and amylopectin were separately measured by identifying them according to the following λ_{max} of *A* of iodine carbohydrate complexes in each tube; amylose, $\lambda_{max} \geqslant 620$ nm, amylopectin, $\lambda_{max} < 620$ nm [23].

Preparation and assay of enzymes. All procedures were performed at 0–4°. Ten grains were hulled, and embryo and pericarp were removed from the hulled rice. Enzymes were extracted from endosperms as reported in Ref. [24]. Enzyme assay were performed as reported in Refs [25, 26] for ADPGlc PPase, Q-enzyme and, soluble and bound starch synthase, and in Ref. [27] for sucrose synthase, respectively. All the enzymes were assayed in a range where the velocity was proportional to the enzyme concn, and the incubation time. Each result is the mean ± s.d. of three replicate incubations for the assay of sucrose synthase and Q-enzyme and four replicate incubations for ADPGlc PPase and soluble and bound forms of starch synthase.

Acknowledgements—This work was financially supported by the Science and Technology Agency of Japan, and the Ministry of Agriculture, Forestry and Fisheries of Japan.

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