

Starch Characteristics of the Rice Mutant *du2-2* Taichung 65 Highly Affected by Environmental Temperatures During Seed Development

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ABSTRACT

Cereal Chem. 80(2):184–187

The effects of environmental temperature (21 vs. 28°C) during rice seed development on the starch characteristics (apparent amylose content, amylopectin chain length distribution, and gelatinization properties) of nonwaxy Taichung 65 (T65), waxy Taichung (T65wx), *du2-2* mutated low-amylose strain Taichung (76-3/T65), and Koshihikari were studied. Amylose contents increased with decreasing environmental temperatures. Analysis of the amylopectin chain length distribution showed that the relative amounts of long chains with degree of polymerization (DP) > 25

in all starches decreased if maturation occurred at 21°C. Gelatinization onset, peak, and conclusion temperatures and enthalpies decreased with decreasing environmental temperatures. Of all starches studied, the *du2-2* mutated low-amylose Taichung (76-3/T65) was most affected by maturation temperatures. These results indicate that the *du2-2* mutated low-amylose Taichung (76-3/T65) may be a useful strain in understanding biochemical and genetic starch biosynthesis response to slight changes in temperature.

The amylose content of endosperm starch is an important characteristic determining the eating and cooking quality (Juliano 1992; Chikubu 1995). Countries have different preferences for rice with varying amylose contents. First, both a single dominant gene with a major effect and several modifying genes with a minor effect determine the amylose content in nonwaxy strains. Rice strains with the *Wx^b* gene, one of the alleles at the rice *waxy* (*wx*) locus, contain 15–20% amylose in contrast to rice strains with the *Wx^a* gene with ≥20–25% amylose (Sano 1984). Levels of *Wx* gene expression and amylose synthesis are controlled by other loci, such as the *du* loci (Satoh and Omura 1981; Okuno et al 1983). The mutation at the *du* locus reduces the production of the *Wx* protein resulting in lower amylose contents in the endosperm (Yano et al 1988; Hirano 1993). Second, the environmental temperature during seed development also affects the amylose synthesis in rice with the *Wx^b* allele. The levels of *Wx* protein increase in lower temperatures, resulting in higher amylose contents in mature seeds (Inatsu 1979; Asaoka et al 1984; Sano et al 1985). Amylose content thus varies with year and site of cultivation, even within the same cultivar (Inatsu 1979; Asaoka et al 1984). It is, therefore, important to evaluate how starch characteristics vary in response to environmental temperatures and to develop rice cultivars with amylose contents that do not change with environmental temperature.

We previously isolated the *du2-2* mutated low-amylose strain 76-3/T65, on the background of Taichung 65 (T65) (Sano 1985; Dung et al 2000) and reported that 76-3/T65 responds to cool temperature in the same way as nonwaxy rice (T65) (Hirano and Sano 1998; Suzuki et al 2002). In this article, we describe further characterization of 76-3/T65 starches matured at different temperatures, comparing with those of nonwaxy T65 and waxy T65, and show that 76-3/T65 was the most affected by maturation temperatures.

MATERIALS AND METHODS

Experimental Plants Used in Environmental Temperature Control

Materials used were Taichung 65 (T65), a Japonica type of *Oryza sativa* from Taiwan, waxy Taichung 65 (T65wx), a near isogenic line (NIL) of T65 with a waxy single recessive gene, and *du2-2* Taichung 65 (76-3/T65), a NIL of T65 carrying a low-amylose mutant gene *du2-2* (Sano 1985; Dung et al 2000). Koshihikari, the most popular cultivar in Japan, was also used. T65 and Koshihikari were nonwaxy rice cultivars with the *Wx^b* gene. After five days of anthesis, rice plants were grown in temperature-controlled growth chambers at 28 or 21°C until seeds matured.

Preparation of Rice Starch

Brown rice for each strain was polished with an experimental grain polisher (Pearlest, Kett, Co., Tokyo, Japan). Each 1 g of endosperm was homogenized in a Polytron processor (Kinematica, Littau-Lucerne, Switzerland) in 10 mL of methanol. The resulting suspension was boiled for 10 min and centrifuged at 2,500 × *g* for 10 min. Precipitate fractions were washed twice with 20 mL of methanol, suspended in 20 mL of 0.1% NaOH, and centrifuged at 2,500 × *g* for 10 min. After the resulting yellowish layer was removed using a spatula, starch fractions were resuspended in 20 mL of 0.1% NaOH and centrifuged. After washing once again in 0.1% NaOH, the precipitate fractions were washed several times with 20 mL of distilled water and three times with 20 mL of acetone. Fractions were dried in a vacuum at room temperature.

Amylose Content

The apparent amylose contents were analyzed based on an iodine colorimetric method (ICM) (Juliano 1971) with slight modifications (Suzuki et al 2002). To each starch sample (20 mg), 0.1 mL of ethanol and 0.9 mL of 1*N* NaOH were added. After boiling for 10 min, 5 mL of distilled water was added and the sample was homogenized using a Polytron processor. After dilution to 10 mL with distilled water, a colorimetric assay was done using potato amylose (Sigma, Type III) and waxy rice (T65wx) starch mixtures as standards. The starch was prepared from T65wx rice collected from an experimental field managed by the National Institute of Crop Science (Ibaraki, Japan). Apparent amylose contents were also determined using an amperometric titration method (ATM) (Kiribuchi-Otobe et al 1997). Starch (200 mg) was gelatinized with 10 mL of 2.5*N* KOH. The solution was made up to 50 mL with distilled water. A 5-mL aliquot was pipetted into a beaker and 40 mL of distilled water, 5 mL of 1*N* HCl, and 2.5 mL of 0.4*N* KI was added. The mixed suspension was titrated with

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0.00157N KIO₃. Potato amylose and waxy rice (T65wx) starch mixtures were used as standards.

HPAEC-PAD of Isoamylase Debranched Starch

Gelatinized starch (5 mg), suspended in 4.96 mL of distilled water and tempered at 100°C for 6 min, was added to 100 µL of 1M acetate buffer (pH 3.5) and 10 µL of *Pseudomonas amylo-deramosa* isoamylase (10 µg protein/10 µL, 590 units/µg of protein, Hayashibara Biochemical Laboratories, Okayama, Japan), and incubated at 45°C for 2.5 hr. The reaction mixture was added to 200 µL of 1N NaOH and filtered through a 0.22-µm filter (Millipore, Bedford, MA) (Nagamine and Komae 1996). Isoamylase-debranched polyglucan samples were analyzed using high-performance anion-exchange chromatography pulsed amperometric detection (HPAEC-PAD) (Dionex, Sunnyvale, CA). Data were obtained by calculating the sum of peak areas corresponding to fa fraction (DP ≤ 12), fb₁ (13 ≤ DP ≤ 24), fb₂ (25 ≤ DP ≤ 36), and fb₃ (37 ≤ DP) (Hanashiro et al 1996). All analyses were conducted in duplicate.

DSC

Differential scanning calorimetry (DSC) measurements were conducted (SSC 5200, DSC 120U, Seiko Electronics, Tokyo, Japan) and calibrated with indium as previously reported (Sasaki and Matsuki 1998). For gelatinization studies, 50 mg of 10% starch suspension (w/v) was weighed into silver pans. After sealing, pans were scanned at 1°C/min from 40 to 120°C. All DSC analyses were conducted in duplicate.

Statistical Analysis

Data were analyzed using analysis of variance. When statistically significant effects of strains on specific items were found, differences among items were tested using protected least square differences (post hoc test).

RESULTS AND DISCUSSION

The amylose content in endosperm starch was determined by an iodine colorimetric method (ICM) and amperometric titration method (ATM) (Table I). The amylose content of nonwaxy T65 matured at a high temperature (28°C) was 18% for ICM and 13% for ATM, and that matured at a cool temperature (21°C) was 23% for ICM and 22% for ATM. Although precise amylose content differed between analytical methods, maturation temperatures greatly influenced the amylose content of T65 with the *Wx^b* gene and the amylose difference between maturation temperatures was 5% for ICM and 9% for ATM. The amylose difference of Koshihikari matured at different temperatures was ≈6% for both methods. The amylose content of *du2-2* mutant 76-3/T65 matured at 28°C was 4% for ICM and 1% for ATM, lower than 4% and almost identical to that of T65wx. In contrast, the amylose content of 76-3/T65 matured at 21°C was 15% for ICM and 13% for ATM, 10% or higher than that at 28°C and intermediate between T65 and T65wx. Amylose content of 76-3/T65 endosperm was thus more affected by temperature during ripening than nonwaxy rice T65 and Koshihikari.

In addition to the effect of environmental temperature on the amylose content, we should account for the observed differences in amylose contents between the two methods. ICM is a rapid and simple method to measure many samples but it is not accurate (Juliano 1971; Baba 1986). Absorbance readings at 620 nm by ICM also gave lower values for higher amylose samples and higher values for very low amylose samples (Banks et al 1974; Baba 1986). Because the ATM method is the most accurate method for determining the iodine affinity with amylose (Baba 1986), the differences between the two methods may be responsible for the tendency of ICM to overestimate amylose content because of long amylopectin chains. To ascertain whether amylopectin chain length distribution in rice

endosperm is also affected by temperature during seed development, we conducted HPAEC-PAD using isoamylase-debranched materials of rice starch. Consistent with published reports (Asaoka et al 1984; Umemoto et al 1999; Inouchi et al 2000), lower environmental temperatures (21°C) decreased the relative amount of long amylopectin chains (fb₂ and fb₃ fractions) and increased the relative amount of short chains (fa fractions) compared with higher temperatures for all strains (Table II), suggesting that synthesis of amylopectin is affected by maturation temperatures. No apparent differences in relative amounts of amylopectin short chains (fa and fb₁) were found in the three Taichung strains as well at 21 or 28°C. While the relative amounts of fb₃ (long chains) matured at 28°C were almost the same among the three strains, the relative amount of fb₃ matured at 21°C significantly differed; that is, the higher the apparent amylose content, the higher the relative amount of fb₃. Because the amylopectin of mutants lacking *Wx* protein has a shorter average chain length than that of the equivalent wild type (Hizukuri et al 1989), and there have been many reports showing that the *Wx* protein is also involved in the synthesis of long amylopectin chains (Fulton et al 2002), the differences in the relative amount of fb₃ matured at 21°C suggest that *Wx* protein can also synthesize long chains in amylopectin.

Table III shows differential scanning calorimetry gelatinization characteristics of endosperm starch. Lower environmental temperatures during seed development significantly decreased onset (*T_o*), peak (*T_p*), and conclusion (*T_c*) gelatinization temperatures, and gelatinization enthalpy (ΔH) of all starches, as reported elsewhere (Asaoka et al 1984). The decreased gelatinization characteristics at 21°C were probably caused by the increased amylose contents (Table I) and short chains of amylopectin (fa and fb₁) and by decreased long chains of amylopectin (fb₂ and fb₃) (Table II). The gelatinization temperatures and enthalpy of 76-3/T65 matured at 28°C were almost the same as those of T65wx and much higher than those of nonwaxy T65. These results were consistent with the characteristics of strains for the amylose content, specifically, the amylose content of 76-3/T65 was almost identical to that of T65wx and 12% or lower than that of T65 (Table I). At 21°C, however, the gelatinization characteristics of 76-3/T65 were intermediate between those of T65 and T65wx, corresponding to the amylose content, in which 76-3/T65 was also intermediate between T65 and T65wx. Accordingly, the gelatinization characteristics were attributed to the amylose content and the chain length distribution of amylopectin, with the characteristics of the *du2-2* mutated Taichung (76-3/T65) starch being more affected by maturation temperatures than those of nonwaxy T65 or waxy T65wx.

TABLE I
Apparent Amylose Content of Starches from Rice Plants
Matured at 28 or 21°C

Temperature	Strain	Amylose Content (%) ^a	
		Iodine Colorimetric Method	Amperometric Titration Method
28°C	T65	18.1 ± 0.8a	13.4 ± 1.2a
	76-3/T65	3.6 ± 0.4b	0.7 ± 0.0b
	T65wx	1.6 ± 0.2c	0.2 ± 0.2b
	Koshihikari ^b	14.4 ± 0.3	9.5 ± 0.6
21°C	T65	23.1 ± 0.1a	22.2 ± 1.1a
	76-3/T65	14.7 ± 0.4b	12.5 ± 1.2b
	T65wx	-1.2 ± 0.0c	0.4 ± 0.3c
	Koshihikari ^b	20.4 ± 1.2	16.0 ± 1.9
Significance of treatments ^c			
	Temperatures	**	**
	Strain	**	**
	Interaction	**	**

^a Means followed by the same letter within the same temperature column did not differ significantly at 5% in protected least square differences.

^b Measured values of Koshihikari references not included in statistics analyses.

^c **, significant at 1%.

TABLE II
Amylopectin Chain Length Distribution of Starches from Rice Matured at 28 or 21°C

Temperature	Strain	Chain Length Distributions of Amylopectin (%) ^{a,b}			
		fa	fb ₁	fb ₂	fb ₃
28°C	T65	26.0 ± 0.2	45.4 ± 0.1	12.0 ± 0.1a	16.7 ± 0.3a
	76-3/T65	26.0 ± 0.3	45.5 ± 0.4	12.1 ± 0.1a	16.4 ± 0.2a
	T65 _{wx}	26.4 ± 0.3	45.9 ± 0.6	11.9 ± 0.1a	15.8 ± 0.4a
	Koshihikari ^c	26.1 ± 0.2	45.6 ± 0.2	12.1 ± 0.1	16.3 ± 0.0
21°C	T65	28.1 ± 0.1	46.2 ± 0.3	11.2 ± 0.0a	14.6 ± 0.1a
	76-3/T65	28.5 ± 0.1	46.7 ± 0.1	11.2 ± 0.1a	13.7 ± 0.1b
	T65 _{wx}	28.2 ± 0.0	46.8 ± 0.1	11.8 ± 0.1b	13.2 ± 0.1c
	Koshihikari ^c	27.3 ± 0.0	46.5 ± 0.1	12.0 ± 0.1	14.4 ± 0.0
Significance of treatments ^d	Temperature	**	**	**	**
	Strain	ns	ns	*	**
	Interaction	ns	ns	**	ns

^a Degrees of polymerization: fa, DP ≤ 12; fb₁, 13 ≤ DP ≤ 24; fb₂, 25 ≤ DP ≤ 36; fb₃, 37 ≤ DP.

^b Means followed by the same letter within the same temperature column did not differ significantly at 5% in protected least square differences.

^c Measured values of Koshihikari references were not included in statistics analyses.

^d **, *, significant at 1 and 5%; ns, not significant.

TABLE III
Differential Scanning Calorimetry of Starches from Rice Plants Matured at 28 or 21°C

Temperature	Strain	Gelatinization Characteristics ^{a,b}			
		T _o	T _p	T _c	ΔH
28°C	T65	52.9 ± 0.5a	59.7 ± 0.4a	69.2 ± 0.1a	14.1 ± 0.1a
	76-3/T65	54.6 ± 0.2b	64.3 ± 0.1b	73.3 ± 0.3b	15.9 ± 0.0b
	T65 _{wx}	54.8 ± 0.3b	63.6 ± 0.2b	76.8 ± 0.1c	15.9 ± 0.5b
	Koshihikari ^c	53.3 ± 0.3	60.2 ± 0.1	68.7 ± 0.1	14.6 ± 0.4
21°C	T65	43.1 ± 0.1a	52.1 ± 0.0a	58.5 ± 0.1a	11.9 ± 0.0a
	76-3/T65	44.5 ± 0.4b	52.8 ± 0.1b	61.5 ± 0.6b	12.3 ± 0.1b
	T65 _{wx}	45.3 ± 0.4b	55.5 ± 0.1c	68.2 ± 1.1c	14.2 ± 0.1c
	Koshihikari ^c	45.5 ± 0.1	53.4 ± 0.1	60.7 ± 0.2	12.3 ± 0.1
Significance of treatments ^d	Temperature	**	**	**	**
	Strain	**	**	**	**
	Interaction	**	**	ns	**

^a Gelatinization temperatures (°C): T_o, onset; T_p, peak; T_c, conclusion; ΔH, enthalpy (J/g).

^b Means followed by the same letter within the same temperature column did not differ significantly at 5% in protected least square differences.

^c Measured values of Koshihikari references were not included in statistics analyses.

^d **, *, significant at 1%; ns, not significant.

The amylose content, chain length distribution, and gelatinization characteristics of 76-3/T65 starch matured at 28°C were similar to those of T65_{wx}. Those of 76-3/T65 matured at 21°C, however, differed from T65 and T65_{wx}, *du2-2* Taichung (76-3/T65), and waxy Taichung (T65_{wx}) are NIL of T65, except that they carry either *waxy* or *du2-2* alleles. Variation in the structures and properties of these starches should derive from the differences in alleles.

Thus far, five nonallelic *du* mutants with dull endosperms were isolated and all were recessive and nonallelic to the *waxy* locus (Yano et al 1988). In this study, we used the low-amylose strain *du2-2* 76-3/T65. The mutant gene *du2-2* of 76-3/T65 was allelic to *du2*, and the phenotypes (amylose contents and endosperm transparency) of 76-3/T65 were distinct from those of *du2* mutant (Yano et al 1988; Dung et al 2000). The *du2* (Isshiki et al 2000) and *du2-2* (Dung et al 2000) mutations do not affect the processing of *Wx^d* pre-mRNA after splicing and the *Wx* protein level of *Wx^d* rice, respectively. However, *du2-2* reduces the amylose content of *Wx^d* rice (Dung et al 2000). Therefore, whether the starch characteristics of *du2-2 Wx^d* rice are affected by the maturation temperatures similar to those of *du2-2 Wx^b* rice (76-3/T65) or not is a subject for further study.

The amylose content of rice endosperm is a major determinant of eating quality because amylose content profoundly affects the stickiness of cooked rice (Webb 1991; Chikubu 1995). Japanese consumers generally prefer rice with lower amylose contents because of the viscous texture of the cooked rice. Amylose content, however, varies from year to year with the location of the same cultivar (Asaoka et al 1984; Inatsu 1979; Okuno et al 1993). The amylose

content of nonwaxy T65 and Koshihikari rice fluctuated 6%, whereas 76-3/T65 fluctuated 10% depending on maturation temperatures (Table I). Because low-amylose cultivars (5–15% amylose content) compared to nonwaxy (wild type) cultivars (15–20%) have been bred in Japan (Sato 2002), it is important to find out to what extent starch characteristics are affected by maturation temperatures, as well as to develop rice cultivars that are insensitive to maturation temperatures. As shown in Tables I–III, the starch characteristics of 76-3/T65 were most affected by maturation temperature. Consequently, *du2-2* Taichung (76-3/T65) may be a useful strain in understanding biochemical and genetic starch biosynthesis response to slight changes in temperature.

ACKNOWLEDGMENTS

We thank K. Komae (Natl. Inst. Crop Sci.) and T. Kato (Tochigi Agric. Exp. Stn.) for invaluable advice and S. Sakurai (Natl. Inst. Crop Sci.) for technical assistance.

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[Received April 30, 2002. Accepted November 24, 2002.]