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Soluble Starch Synthase I Effects Differences in Amylopectin Structure between *indica* and *japonica* Rice Varieties

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The effect of soluble starch synthase I (SSI) on differences of amylopectin structure between the *indica* and *japonica* rice varieties was investigated. Native-PAGE/active staining analysis showed that the SSI activity of an *indica* rice variety, 'Kasalath', was significantly lower than that of a *japonica* rice variety, 'Nipponbare', and that the low activity in 'Kasalath' was maintained during seed development. The result of northern blot analyses suggests that the low expression of SSI in 'Kasalath' is controlled at the transcription levels of SSI mRNA. Chain length distribution of amylopectin in F_3 endosperms derived from a cross between two varieties showed that not only SSIIa but also SSI regulated the population of short chains. These results indicate that the low activity of SSI gives rise to the decrease of short chains in amylopectin of *indica* rice varieties, suggesting that SSI effects the differences in physicochemical properties between two varieties.

KEYWORDS: Soluble starch synthase I; *indica* rice variety; *japonica* rice variety; amylopectin

INTRODUCTION

Starch serves a fundamental role in the life cycle of plants as the carbohydrate storage substance and the most important source of calories in the human diet. Rice (*Oryza sativa* L.) is one of the most important food crops in the world. Especially in Asia, rice is a crop that is widely used as a staple food and is used for various industrial applications. Rice varieties are classified into two subspecies, *japonica* and *indica*, based on the morphological and serological character as well as intervarietal fertility (*1*) and isozymes such as esterases (*2*). The quantity and quality differences in seed storage components effect differences in the eating quality of cooked rice between *indica* and *japonica* rice varieties. The physicochemical property of cooked rice is one of the most important factors for eating quality. The physicochemical property of cooked rice is mainly influenced by the rice starch deposited in the endosperm.

Rice starch consists of amylose, which is essentially a linear molecule composed of α-1,4-linked glucosidic chains and amylopectin, which is a highly branched glucan with α-1,6-glucosidic bonds. The synthesis of amylopectin begins with the formation of the activated glucosyl donor ADP glucose (ADPG), catalyzed by ADPG pyrophosphorylase (AGPase). Reactions to build α-1,4-linked linear glucosyl chains using ADPG are catalyzed by the soluble starch synthase (SSS). The branched structure of amylopectin was formed by branching enzyme (BE) and debranching enzyme (DBE) (*3*).

The physicochemical property of cooked rice is mainly influenced by the amylose content. However, recent research has shown that the amylopectin structure also greatly influences the physical properties of starch (*4*−*6*). Mutations in BE genes cause specific alterations in the amylopectin structure and physicochemical properties of starch (*5*). A BE I-deficient mutation altered amylopectin fine structure and caused a lower onset concentration for urea gelatinization and a lower onset temperature for thermogelatinization compared with the wild type (*6*). Thus, modification of amylopectin fine structure caused by the changes in expression of starch-synthesizing enzymes is responsible for changes in the physicochemical properties of rice starch.

Four isoforms of SS have been identified in higher plants, including SSI, SSII, SSIII, and SSIV. Analyses of rice mutant with retrotransposon inserted into the SSI gene suggest that the function of SSI is mainly involved in the synthesis of short chains of degree of polymerization (DP) of ≤12 of amylopectin (*7*). The lack of SSII activity in the pea mutant *rug5* and barley mutant *sex6* results in a loss of intermediate length chains of amylopectin, indicating that SSII synthesizes intermediate chains of $12 \leq DP \leq 24$ of amylopectin (*8*, *9*). SSIII produces longer chains extending between clusters (*10*). Detail of the roles of SSIV for amylopectin synthesis remain unknown. Although these results suggest that these SSs play a distinct role in the amylopectin synthess, nevertheless, the effect of SS activity on the physicochemical properties of starch are not well understood.

Nakamura et al. (*11*) reported four groups of rice cultivars, *indica*, Chinese *indica*, temperate *japonica*, and tropical *japon-*

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ica, based on the pattern of esterase isozyme and physiological and biochemical features of seeds and classified into two classes by the differences of fine structure of amylopectin. According to the comparison in two classes, amylopectin from *indica* rice was remarkably depleted in short chains of (DP) ≤11 and enriched in intermediate chains of 12 ≤ DP ≤ 24 as compared with that from *japonica* rice (*11*, *12*). The differences in the expression level of SSIIa correlate with the alkali gelatinization property between the two varieties (*12*, *13*). These results show that the difference of the gelatinization property was caused by the difference of the amylopectin structure in both varieties. These results imply that SSIIa, in addition to GBSS, is one of the key enzymes that cause the differences of physicochemical properties of rice starch between the two varieties. It remains to be examined whether other starch-synthesizing enzymes effect differences in physicochemical properties of starch between *indica* and *japonica* rice varieties or not.

In this paper, we report that the differences of the expression level of the SSI effect the differences of the chain length distribution between the *indica* rice variety 'Kasalath' and the *japonica* rice variety 'Nipponbare'. For this purpose, the expression level of SSI activities in both varieties was investigated. To show the influence of SSI on the fine structure of amylopectin, the chain length distribution of amylopectin in endosperm starch obtained by F_3 from a cross between 'Kasalath' and 'Nipponbare' was determined by HPAEC-PAD. The multiple regression analysis using SSI and SSIIa as variables showed that not only SSIIa but also SSI affects the ratio of short chains of amylopectin. These results show that the differences of SSI activity also caused the differences of amylopectin structure between the *indica* and *japonica* rice varieties.

MATERIALS AND METHODS

Plant Materials. Rice varieties 'Kasalath' (*O. sativa* L. *indica*), 'IR36' (*O. sativa* L. *indica*), 'Nipponbare' (*O. sativa* L. *japonica*), and 'Koshihikari' (*O. sativa* L. *japonica*) were grown in plastic pots in a growth chamber. The developing seeds were used in biochemical studies. F_2, F_3, and F_4 populations derived from a cross between 'Kasalath' and 'Nipponbare' were prepared. F_2 seeds were prepared for the calibration model for the ratio of short chains of DP ≤ 12 to intermediate chains of DP ≤ 24 on the basis of the genotype of SSI and SSIIa. F_3 seeds were prepared for determination of the distribution of lengths of α-1,4-glucan chains in α-polysaccharides by high-performance anion exchange column pulsed amperometoric detection (HPAEC-PAD). F_4 populations were prepared for alkali gelatinization property as described by Umemura et al. (*13*).

Activity Staining of SS on Native-PAGE. An immature rice grain was removed from the hull and pericarp and weighed. Dehulled seed was homogenized with a mortar and pestle on ice within the same weight of solution, which consisted of 50 mM HEPES−NaOH (pH 7.4), 2 mM $MgCl_2$, 50 mM 2-mercaptoethanol, and 12.5% (v/v) glycerol (*5*). The homogenate was centrifuged at 15000*g* for 15 min. The supernatant was used as the crude enzyme extract. Native-PAGE was performed on a slab gel prepared with 7.5% (w/v; resolving gel) containing 0 or 0.8% (w/v) oyster glycogen (type II; Sigma) and 3.3% (w/v; stacking gel) acrylamide according to a modified version (*5*) of the method described by Davis (*14*). Electrophoresis was carried out at 4 °C. After electrophoresis, the gel was rinsed with 35 mL of a solution of 100 mM Bicine−NaOH buffer (pH 7.5), 0.5 mM EDTA, 2 mM DTT, and 10% (v/v) glycerol and either in the presence or in the absence of 0.5 M citrate−sodium buffer (pH 7.5) for 15 min on ice, and then it was incubated for 12 h at 30 °C in 35 mL of the reaction mixture, which consisted of 100 mM Bicine−NaOH buffer (pH 7.5), 0.5 mM EDTA, 2 mM DTT, 10% (v/v) glycerol, and 1 mM ADP-Glc and either in the presence or in the absence of 0.5 M citrate−sodium buffer (pH 7.5). The gel was placed in an iodine solution that consisted of 0.1% (w/v) I_2 and 1% (w/v) KI (*5*).

Northern Blotting Analysis. Total RNA was extracted from developing seeds by using the SDS−phenol method according to the process described by Kim and Okita (*15*). Extracted RNA was electrophoresed on a gel prepared with 1.2% (w/v) agarose gel containing MOPS and 6.5% (v/v) formaldehyde and was blotted onto positively charged nylon membranes (Amersham Pharmacia Biotech, Piscataway, NJ) (*15*). cDNA was obtained by using a T-primed First strand kit (Amersham Biosciences). The cDNA fragment for SSI or GBSS primer was obtained by PCR with SSI primer (5′-AGG′ GGT′ ATT′ CAT′ GGG′ AGG′ TC-3′ and 5′-ATG′ GTT′ AGT′ GGC′ GAG′ AAT′ GC-3′) and GBSS primer (5′-GAG′ GTC′ CTG′ GTT′ CAT′ GCA′ GT-3′ and 5′-GGC′ AAG′ ACT′ GGT′ TTC′ CAC′ ATC-3′) labeled using the enhanced chemiluminescence labeling system (Amersham Pharmacia Biotech).

Restriction Fragment Length Polymorphism (RFLP) Analysis. Genomic DNA was extracted from F_3 leaves and parental lines according to the CTAB method (*16*), and SSI primer was used for PCR. In the PCR condition, the annealing is performed at 57 °C for 1 min and the extension reactions are performed at 72 °C for 2 min. Each PCR product was digested by a restriction enzyme *Eco*RV (Takara Co. Ltd.), and electrophoresis was performed on a 1.4% agarose gel.

Determination of the Distribution of Lengths of α-1,4-Glucan Chains in α-Polysaccharides by HPAEC-PAD. Half of the F_3 seeds were ground with a mortar and pestle, and 5 mg of the resulting powder was suspended in 5 mL of methanol and boiled for 10 min. The homogenate was centrifuged at 2500*g* for 5 min. The pelleted starch was washed twice with 5 mL of distilled water, suspended in 5 mL of distilled water, and then boiled for 60 min. An aliquot (1.0 mL) of the sample of gelatinized starch was added to 50 μL of 600 mM sodium acetate buffer (pH 4.4) and 10 μL of 2% (w/v) NaN_3, and then hydrolysis was achieved by the addition of 1400 units of isoamylase (Hayashibara Co.) and incubation at 40 °C for 24 h. The hydroxyl groups of the debranched glucans were reduced by treatment with 0.5% (w/v) of sodium borohydride under alkaline conditions for 20 h according to the method of Nagamine and Komae (*17*). The preparation was dried by freeze-dryer and allowed to dissolve in 100 μL of 1 M NaOH for 60 min. Then, the solution was diluted with 900 μL of distilled water. An aliquot (25 μL) of the preparation was injected into a BioLC (System model DX-500, Dionex, Sunnyvale, CA) equipped with a PAD and a Carbopac PA-1 column (4 mm i.d. × 25 cm). Size fractionation of α-1,4-glucans was performed with a linear gradient of sodium acetate (50−500 mM) in 0.1 M NaOH at a flow rate of 1 mL min^{-1}.

RESULTS

The activity of soluble starch synthase I of *indica* rice variety 'Kasalath' was lower than that of *japonica* rice variety 'Nipponbare'. To show the differences of expression level of soluble starch synthases between *indica* rice variety 'Kasalath' and *japonica* rice variety 'Nipponbare', native-PAGE/active staining analysis was carried out in three conditions: in the presence of glycogen and 0.5 M citrate, in the presence of glycogen and in the absence of 0.5 M citrate; and in the absence of glycogen and in the presence of 0.5 M citrate, because SSs are divided into two types according to whether the glucan primer is needed for the first reaction of the starch synthesis. SSI was distinguished from the band pattern of the native-PAGE/active staining reported by Nishi et al. (*5*) and the molecular weight of this band shown by SDS-PAGE (*18*). The activities of SSI were distinctly lower in 'Kasalath' than in 'Nipponbare' in the presence of oyster glycogen as glucan primer and 0.5 M citrate buffer (**Figure 1A**, open arrowhead). Especially, the SSI activity band in 'Kasalath' was hardly detectable (**Figure 1A**). The reduction of SSI activity was also observed in the presence of the glycogen and in the absence of 0.5 M citrate (**Figure 1B**). In the absence of glucan primer and in the presence of 0.5 M citrate, the SSI activity was also detected, the same as in the case of SSI from maize endosperm

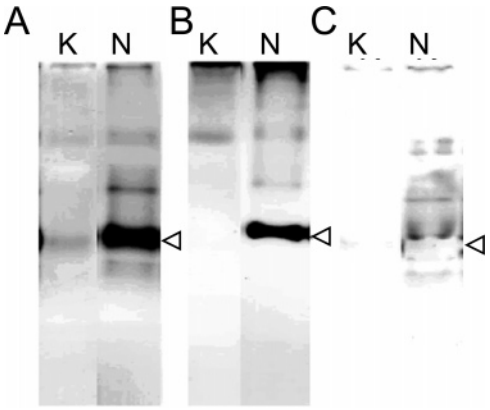

Figure 1. Native-PAGE/activity staining of SSs in the endosperm of *indica* rice variety 'Kasalath' (K) and *japonica* rice variety 'Nipponbare' (N): (**A**) + glycogen and + citrate; (**B**) + glycogen and − citrate; (**C**) − glycogen and + citrate. Each lane contained 10 μL of the crude enzyme extract.

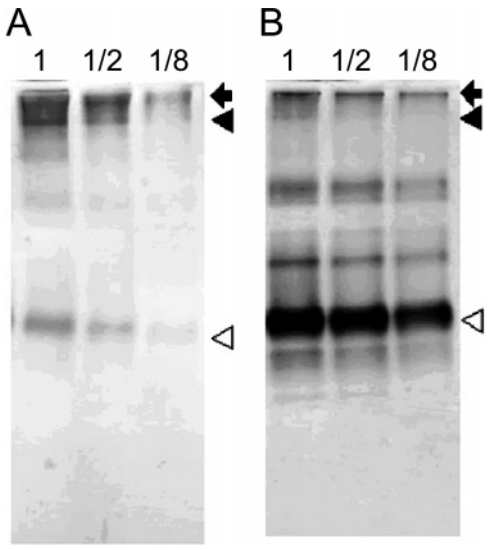

Figure 2. Native-PAGE/activity staining of SSs in the endosperm of *indica* rice variety 'Kasalath' (**A**) and *japonica* rice variety 'Nipponbare' (**B**). Each lane contained 10 μL multiplied by the number given above the lane of the crude enzyme extract.

(*19*). This glucan primer independent SSI activity was also markedly lower in 'Kasalath' than in 'Nipponbare' (**Figure 1C**). The activity of SSI in 'Kasalath' was $^1/_{16}$ or less of that of 'Nipponbare' (**Figure 2**, open arrowhead). Besides the SSI band, some native-PAGE/active stained bands were detected (**Figure 2**). SS activity in 'Kasalath' was higher than that in 'Nipponbare' in the band shown by the solid arrowhead. On the other hand, the activity level of SS in the band shown by the arrow was almost the same between the two varieties. Thus, the expression level of SSs isoform was greatly different between the varieties.

The decrease of SSI activity of 'Kasalath' was maintained during seed maturation. In an attempt to show the expression level of SSI during seed maturation, the activity of SSI extracted from developing seed was investigated. The activity of SSI in 'Nipponbare' was detected 2 days after flowering (DAF), and it increased from 6 to 8 DAF and then remained constant until early dough stage (20 DAF) (**Figure 3B**). After late dough stage, the activity of SSI was decreased with seed maturation, because extracted soluble protein decreased remarkably by dehydration from the starch granule during seed maturation (data not shown). The activity of SSI was detected until the yellow ripe stage,

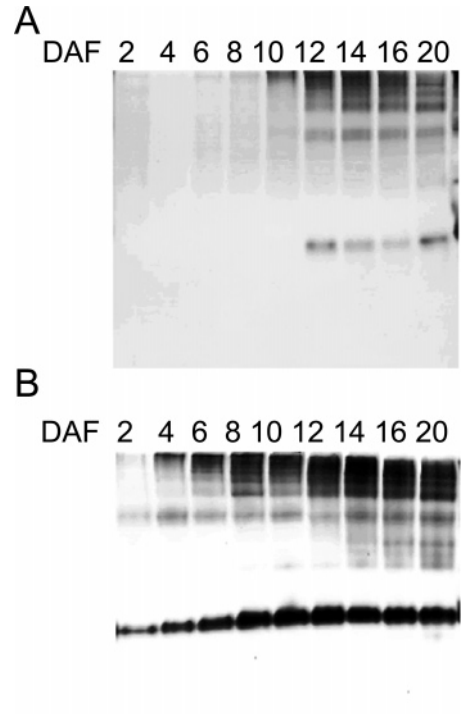

Figure 3. Change of activity level of SSs during seed development: (**A**) 'Kasalath'; (**B**) 'Nipponbare'. Each lane contained 10 μL of the crude enzyme extract.

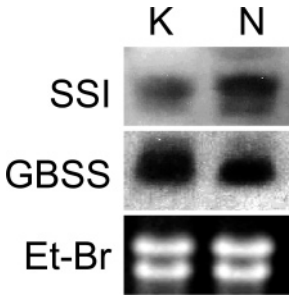

Figure 4. Northern blot analyses of SSI and GBSS transcripts in *indica* rice variety 'Kasalath' and *japonica* rice variety 'Nipponbare'. Total RNA extracted from 15 DAF developing seeds was blotted and proved with DNA probe. The lower portion of the figure shows the ethidium bromide-stained RNA gel.

indicating that SSI in 'Nipponbare' showed strong activity during seed maturation. On the other hand, in 'Kasalath', SSI activity was detected from the milky stage (**Figure 3A**). The SSI activity in 'Kasalath' was significantly lower than that of 'Nipponbare'. These results showed that the decrease of SSI activity was maintained during seed maturation.

The low expression level of SSI in 'Kasalath' is controlled at the transcription levels of SSI mRNA. To confirm whether the expression of SSI is suppressed at the transcript level in 'Kasalath', we further investigated the RNA isolated from developing seeds of 'Kasalath' and 'Nipponbare' by northern blot analysis using the rice SSI cDNA as a probe. The probe reacts with both varieties, but the reaction intensity of 'Kasalath' was lower than that of 'Nipponbare' (**Figure 4**). GBSS mRNA was detected in 'Kasalath' and 'Nipponbare' at almost the same densities (**Figure 4**). These results suggest two possibilities. One is that the low expression level of SSI in 'Kasalath' is caused by a gene of SSI, meaning that expression of SSI is controlled at the transcription level of SSI mRNA. The other possibility is that the decrease of expression level of RNA in 'Kasalath'

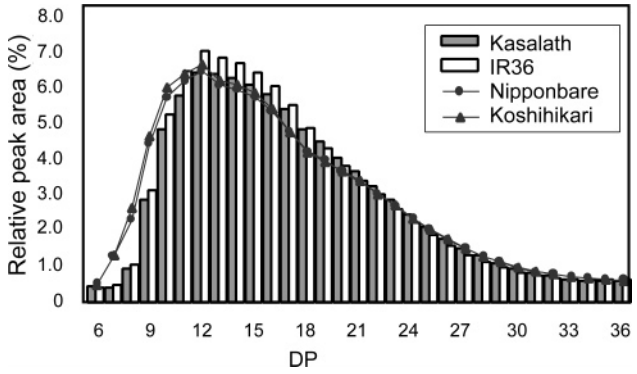

Figure 5. Distribution of chain length distribution of total α-polysaccharides from the endosperm starch of *indica* rice variety 'Kasalath' and 'IR36' and that of *japonica* rice varieties 'Nipponbare' and 'Koshihikari' as determined by HPAEC-PAD. Amylopectin was debranched by isoamylase and reduced with sodium borohydride. The distribution of reduced α-polysaccharides was fractionated on a Carbopac PA1 column. The α-1,4-glucan chains were eluted with a gradient of sodium hydroxide and sodium acetate and monitored with a PAD.

was influenced by the expression level of other enzymes, just as SSI activity was suppressed in the *ae* mutant (*5*).

The short chains of DP ≤ 12 of amylopectin were synthesized by SSI. To clarify the effect of SSI on the differences of amylopectin structure between the *japonica* and *indica* rice varieties, we prepared the F_3 and F_4 populations from a cross between 'Nipponbare' and 'Kasalath'. The genotype of SSI of each F_3 plant was investigated by RFLP analysis. Furthermore, the chain length distribution of amylopectin in F_3 endosperm starch was determined by HPAEC-PAD.

Chain length distribution of amylopectin in endosperm starch derived from the *indica* rice varieties 'Kasalath' and 'IR36' and from the *japonica* rice varieties 'Nipponbare' and 'Koshihikari' showed that the amylopectin from *indica* rice varieties is markedly depleted in chains 6 ≤ DP ≤ 12, the A chain of amylopectin, and enriched in chains of 13 ≤ DP ≤ 24, the B1 chain of amylopectin, as compared with those from *japonica* rice varieties as reported by Umemoto et al. (*11*) (**Figure 5**).

The F_3 genotype SSI of 'Kasalath' (F_3SSI^{II}) and the F_3 genotype SSI of 'Nipponbare' (F_3SSI^{JJ}) were screened from the F_3 population by using RFLP. Seventeen F_3SSI^{II} plants and 8 F_3SSI^{JJ} plants were selected from 60 F_3 plants. F_3 endosperms were classified into three groups based on chain length distribution of amylopectin. Group 1 was almost similar to the chain length distribution of amylopectin from 'Nipponbare', enriched in chains of 6 ≤ DP ≤ 12 and depleted in chains of 13 ≤ DP ≤ 24 (**Figure 6A**). F_3SSI^{JJ} was included in group 1. Group 3 was similar to that from 'Kasalath'. F_3SSI^{II} was included in group 3 (**Figure 6C**). Group 2 was an intermediate type between 'Kasalath' and 'Nipponbare' and contained both genotypes (**Figure 6B**).

The differences in amylopectin structure between *indica* and *japonica* rice varieties were affected by the activity of SSIIa (*7*, *13*). It is considered that the proportion of short chain was also affected by the activity of SSIIa, because SSIIa plays a role in the synthesis of chains of 13 ≤ DP ≤ 24. Gene-mapping analysis showed that the SSIIa gene is located at the *alk* locus on chromosome 6 in the rice genome (*13*). The genotype of SSIIa of the F_3 population was investigated by alkali gelatinazation of F_4 seeds. All F_3 in group 1 were positive in the alkali gelatinazation test, indicating that the genotype of SSIIa was *japonica* ($SSIIa^{JJ}$). On the other hand, all F_3 in group 3 were negative in the alkali gelatinazation test, showing that the

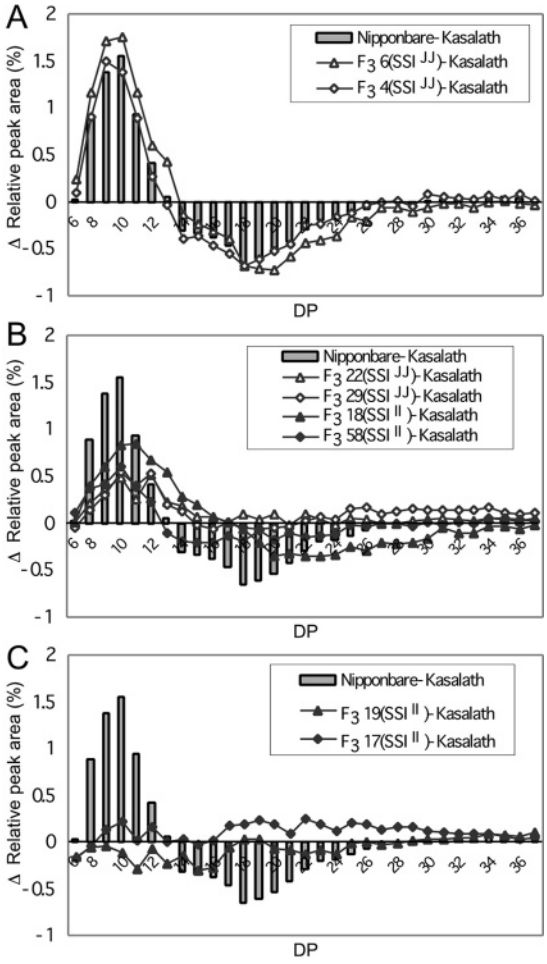

Figure 6. Comparison of chain length distribution of α-polysaccharides from the endosperm starch of F_3. F_3 population was classified into three groups, similar to 'Nipponbare' type (**A**), intermediate type (**B**), and 'Kasalath' type (**C**).

Table 1. Classification of Amylopectin by the Ratio of Short Chains of DP12 to Short and Intermediate Chains of DP ≤ 24

F_3	genotype SSI	genotype SSIIa	ΣDP ≤ 12/ ΣDP ≤ 24	F_3	genotype SSI	genotype SSIIa	ΣDP ≤ 12/ ΣDP ≤ 24
parent varieties							
Kasalath	II	II	0.281				
Nipponbare	JJ	JJ	0.346				
group I				group III			
F_3 4	JJ	JJ	0.345	F_3 7	II	II	0.297
F_3 6	JJ	JJ	0.354	F_3 9	II	II	0.294
F_3 10	JJ	JJ	0.348	F_3 17	II	II	0.287
F_3 26	JJ	JJ	0.344	F_3 19	II	II	0.297
F_3 49	JJ	JJ	0.330	F_3 24	II	II	0.283
group II				F_3 31	II	II	0.291
F_3 22	JJ	II	0.320	F_3 42	II	II	0.284
F_3 29	JJ	II	0.309	F_3 47	II	II	0.284
F_3 41	JJ	II	0.308	F_3 56	II	II	0.293

genotype of SSIIa was *indica* ($SSIIa^{II}$). All F_3 in group 2 were recombinants of the genotypes of SSI and SSIIa.

In the case of F_3SSIIa^{II}, the amylopectin derived from $F_3SSI^{JJ}SSIIa^{II}$ endosperm (**Table 1**, group 2) was increased in chains of 6 ≤ DP ≤ 12 as compared with those from $F_3SSI^{II}SSIIa^{II}$ endosperm (**Table 1**, group 3). These results show that SSI plays an important role in the synthesis of short chains of DP ≤ 12 of amylopectin and that the SSI genotype effects the differences of amylopectin structure between the two varieties. Furthermore, amylopectin from group 2 was depleted

Table 2. Calibration Model for the Ratio of Short Chains of DP ≤ 12 to Intermediate Chain of DP24 Based on the Genotype of SSI and SSIIa ($N = 43$)

I. Genotypes of SSI and SSIIa; Ratio of Short Chains of DP12 to Intermediate Chains of DP ≤ 24 of F_2 Population

F_2	genotype SSI	genotype SSIIa	ΣDP ≤12/ ΣDP ≤ 24	F_2	genotype SSI	genotype SSIIa	ΣDP ≤ 12/ ΣDP ≤ 24	F_2	genotype SSI	genotype SSIIa	ΣDP ≤ 12/ ΣDP ≤ 24
3	IJ	IJ	0.33	18	II	IJ	0.31	40	II	II	0.30
4	JJ	JJ	0.34	19	II	II	0.29	41	JJ	II	0.30
5	IJ	JJ	0.32	21	IJ	JJ	0.31	42	II	IJ	0.33
6	JJ	JJ	0.36	22	JJ	II	0.32	43	IJ	IJ	0.27
7	II	II	0.30	23	II	IJ	0.30	44	II	IJ	0.32
8	II	IJ	0.32	24	II	II	0.29	45	IJ	IJ	0.31
9	II	IJ	0.32	25	IJ	II	0.31	46	IJ	IJ	0.29
10	JJ	JJ	0.37	26	JJ	JJ	0.34	47	II	II	0.28
11	IJ	IJ	0.30	27	IJ	II	0.30	48	IJ	II	0.29
12	IJ	II	0.28	29	JJ	IJ	0.32	49	JJ	IJ	0.32
13	IJ	II	0.31	31	II	II	0.29	52	II	JJ	0.31
14	IJ	IJ	0.28	32	JJ	IJ	0.31	58	II	IJ	0.30
15	IJ	IJ	0.30	35	IJ	II	0.27	59	II	II	0.29
16	IJ	JJ	0.30	36	IJ	JJ	0.34				
17	II	II	0.29	39	II	IJ	0.30				

II. Calibration Model

regressor	partial regression coefficient	standardized partial regression coefficient	P value[a]
SSI^{JJ}	0.025	0.44	0.0003**
$SSIIa^{II}$	–0.029	–0.61	0.0000**
$SSIIa^{IJ}$	–0.017	–0.36	0.0117*
$Y = 0.021(SSI^{JJb}) - 0.032(SSIIa^{IIb}) - 0.017(SSIIa^{IJb}) + 0.319$			
squared multiple correlation adjusted for degree of freedom			0.54
multiple correlation coefficient			0.73

[a] **, 1% significant; *, 5% significant. [b] 1 or 0 is substituted for the variable for the genotype agreement or disagreement, respectively.

in chains of DP ≤ 12 and enriched in chains of DP ≤ 24 compared with that from group 1. It is considered that short chains synthesized by SSI were elongated into intermediate chains of 12 ≤ DP ≤ 24 by SSIIa.

To estimate the contribution of SSI and SSIIa to the proportion of short chains, the calibration model for the ratio of short chains of DP ≤ 12 to intermediate chains of DP ≤ 24 on the basis of the genotype of SSI or SSIIa was made by the stepwise forward regression method (F value = 2.0) within the multiple linear regression analysis (**Table 2**). This calibration formula showed a coefficient of determination of 0.73. SSI^{JJ} had a positive correlation for the Σ 12 ≤ DP/Σ DP ≤ 24, whereas $SSIIa^{II}$ had a negative correlation for that. The standardized partial regression coefficients of SSI^{JJ}, $SSIIa^{II}$, and $SSIIa^{IJ}$ were 0.44, −0.61, and −0.36, respectively. This result indicates that the differences in the short chains of 6 ≤ DP ≤ 12 of amylopectin between the two varieties are effected by not only SSIIa but also SSI.

DISCUSSION

In plants, starch is synthesized by starch synthase catalyzing the transfer of the glucosyl moiety from ADP glucose to the nonreducing ends of glucan chains through a α-1,4 linkages. Fontaine et al. suggested that SSII plays a role in the synthesizing of intermediate chains of amylopectin from the analysis of the SSII deletion mutant of *Chlamydomonas* (*22*). In the case of rice, it was reported that SSIIa acted on the formation of the intermediate chain of amylopectin (*7, 13*). Thus, much information about SSII was obtained on the physiological role for starch synthesis of SSII. However, the function of other SS isoforms is not clarified yet. In this study, to clarify the function of SSI, the following two points were investigated. First, the activity of SSI differed remarkably between *japonica* variety 'Nipponbare' and *indica* variety 'Kasalath'. Second, the relationship between the genotype of SSI and the chain length distribution of amylopectin was investigated by using F_3 and F_4 populations derived from a cross between 'Kasalath' and 'Nipponbare'.

The activity of SSI in *indica* rice variety 'Kasalath' was remarkably lower than that in *japonica* rice variety 'Nipponbare' (**Figure 1**). SSI is one of the major enzymes for starch synthesis in rice endosperm. It is considered that the decrease in the activity of SSI affects the synthesis of starch. From the result of chain length distribution analysis of amylopectin from the F_3 endosperm (**Table 1**; **Figure 6**), we concluded that the proportion of short chains of amylopectin was regulated by not only SSIIa but also SSI. The proportion of short chains of amylopectin in F_3SSI^{II} endosperm was significantly lower than that in F_3SSI^{JJ} endosperm. This result suggests two possibilities. The first possibility is that the high ratio of short chains of DP ≤ 12 to intermediate chains of DP ≤ 24 in 'Nipponbare' amylopectin was caused by the decrease of SSIIa activity in 'Nipponbare' endosperm. The other possibility is that the decrease of Σ12 ≤ DP/Σ 24 ≤ DP in 'Kasalath' endosperm was caused by the low activity of SSI.

The population of F_3SSIIa^{II} was classfied into two groups according to chain length distribution. One is intermediate type that contained $F_3SSI^{JJ}SSIIa^{II}$; the other is 'Kasalath' type that contained $F_3SSI^{II}SSIIa^{II}$ (**Table 1**). This result showed that SSI affected strongly the proportion of short chains whether SSIIa was expressed or not, demonstrating that SSI played an important role in the synthesis of short chains of DP ≤ 12 of amylopectin. Multiple linear regression analysis showed that SSI^{JJ} contributed to the increase of Σ12 ≤ DP/Σ24 ≤ DP. $SSIIa^{II}$ was a factor that decreased Σ12 ≤ DP/Σ24 ≤ DP. It is considered that the elongation of a short chain to an intermediate chain by $SSIIa^{II}$ caused the decrease of Σ12 ≤ DP/Σ24 ≤ DP. In contrast, SSI^{JJ} was a factor that increased Σ12 ≤ DP/Σ24 ≤ DP, indicating that SSI contributes to the increase of Σ12 ≤ DP/Σ24 ≤ DP by synthesizing short chains of amylopectin. In SSI-less mutant by retrotransposon, the decrease of a short chain is reported by Nakamura et al. (*7*). The result of chain length distribution of F_3 $SSI^{II}SSIIa^{JJ}$ agreed with that of that SSI-less mutant (data not shown), suggesting that the decrease of a short chain in Kasalath is caused by the decrease of SSI activity. From these results, it is shown that the differences of the population of short chains in amylopectin between the two varieties are effected by not only SSIIa but also SSI.

Amylopectin is a highly branched glucan with α-1,6 glucosidic bonds that connect linear chains. The α-1,4 chains of amylopectin consist of an A chain of DP ≤ 12, B1 chains of 13 ≤ DP ≤ 24, B2 chains of 25 ≤ DP ≤ 36, B3 chains of DP ≤ 37, and a C chain that includes the reducing terminus (*21*−*23*). Hizukuri proposed a cluster model for amylopectin (*21*). In this model, A and B1 chains form a single cluster, whereas B2 and B3 chains extend to two or three clusters, respectively (*21*). The population of short chains of amylopectin affects the physicochemical properties of starch, because A chains play an important role in the formation of the crystalline structure. Jane et al. reported that there is a correlation between the crystalline structure of starch and its rheological properties (*24*). Rice *ae* mutation specifically altered the structure of amylopectin by reducing short chains (*5*). The extent of such change was correlated with the increase of the gelatinization temperature of starch. In contrast, in the rice *sugary* mutant,

which has lost the function of isoamylase, a significant proportion of short chains is disturbed in the construction of amylopectin multiple structure (*25*). Amylopectin in *sugary* mutants was altered to water-soluble phytoglycogen by the change of the amylopectin structure (*25*). Thus, short chains within the clusters might play a critical role in the rheological properties of starch, which suggests that the activity of SSI would influence greatly the physicochemical properties of rice starch SSI. The physicochemical properties of endosperm starch were remarkably different between the *indica* and *japonica* rice varieties. SSIIa is one of the important factors that caused these differences between the two varieties. In the case of SSI, the reduction of SSI activity was also detected in another *indica* rice variety, 'IR36', by native-PAGE/active staining (data not shown), suggesting that the activity level of SSI in *indica* rice varieties is lower than that in *japonica* rice varieties. This suggestion must be verified by analyzing many other *indica* and *japonica* varieties, and the authors are carrying out the successive research.

Furthermore, the mobility of SSI bands of 'Kasalath' detected in native-PAGE gel was slightly higher than that of 'Nipponbare' (**Figures 1** and **2**). Five SSI sequences were retrieved from the NIH genetic sequence database: one clone derived from an *indica* variety (accession no. AY299404) and another four clones derived from *japonica* varieties (accession no. XM_550329, AK109458, AF165890, and D16202) were obtained. Although these clones displayed 99% identity, respectively, only one base was substituted between the *indica* variety and the four *japonica* varieties. It was guanine (2408) in the *indica* clone and adenine in the four *japonica* clones. This basic substitution accompanied the substitution of the amino acid Glu in the *indica* variety into Lys in the *japonica* varieties. Eight conserved sequence motifs of the SS gene were reported by Cao et al. (*26*). The substitution of amino acid existed between the *indica* variety and the four *japonica* varieties was contained in the fourth motif. The role of the fourth motif remains unknown. It is suggested that possibly each SSI enzyme was different in protein properties. Actually, this substitution of amino acid caused the change of isoelectric point on calculation of SSI protein, that is, p*I* 5.70 in *indica* SSI and p*I* 5.86 in *japonica* SSI.

We propose the model concerning the effect that SSI and SSIIa give the differences of amylopectin structure in two varieties based on these pieces of information. In the case of *japonica* rice varieties, the high activity of SSI and the low expression of SSIIa would cause a higher proportion of short chains of *japonica* type amylopectin. On the other hand, in the case of *indica* varieties, the low activity of SSI and the high expression of SSIIa would lead to a higher proportion of intermediate chains of the *indica* type amylopectin. In the same plant starch granule, a high ratio of short chains to long chains affects the rheological properties, such as gelatinization temperature (*27*) and retrogradation (*28*). These results suggested strongly that the ratio of short chains to long chains is an important factor for the physicochemical properties of starch. It is clarified that there are correlations in the change of the ratio of short chains of amylopectin and the change of physicochemical properties of starch from research on the *ae* mutant (*6*), the *sbe1* mutant (*7*), and the *sug* mutant (*25*). A hypothesis that the increase of short chains caused the increase of stickiness of cooked rice starch is suggested. Thus, it is indicated that SSI plays an important role in "sticky" starch, which is a typical starch property of *japonica* rice varieties, and that the high expression of SSIIa caused "nonsticky" starch, which is a typical property of *indica* rice varieties.

In this study, we suggest that the structural differences of amylopectin between the *indica* and *japonica* varieties were caused by the combination of expression of two enzymes, SSI and SSIIa. It is considered that other starch synthesizing enzymes, such as BEs and DBEs, also influence the amylopectin structure. The influence that these enzymes including SSI have on the difference of physicochemical properties of cooked rice between the *indica* and *japonica* rice varieties remains unclear. The hybrid lines between 'Kasalath' and 'Nipponbare' prepared in this study are very useful in clarifying the influence of the difference of the expression level of starch synthesizing enzymes between the two varieties, for which the physicochemical properties of cooked rice are markedly different.

ABBREVIATIONS USED

AGPase, ADP-Glc pyrophosphorylase; DAF, days after flowering; DP, degree of polymerization; GBSS, granule bound starch synthase; SS, soluble starch synthase; BE, branching enzyme; DB, debranching enzyme; HPAEC-PAD, high-performance anion exchange column pulsed amperometoric detection.

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