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Essential amino acids of starch synthase IIa differentiate amylopectin structure and starch quality between *japonica* and *indica* rice varieties

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Abstract

Four amino acids were variable between the 'active' *indica*-type and 'inactive' *japonica*-type soluble starch synthase IIa (SSIIa) of rice plants; Glu-88 and Gly-604 in SSIIa of *indica*-cultivars IR36 and Kasalath were replaced by Asp-88 and Ser-604, respectively, in both *japonica* cultivars Nipponbare and Kinmaze SSIIa, whereas Val-737 and Leu-781 in *indica* SSIIa were replaced by Met-737 in cv. Nipponbare and Phe-781 in cv. Kinmaze SSIIa, respectively. The *SSIIa* gene fragments shuffling experiments revealed that Val-737 and Leu-781 are essential not only for the optimal SSIIa activity, but also for the capacity to synthesize *indica*-type amylopectin. Surprisingly, however, a combination of Phe-781 and Gly-604 could restore about 44% of the SSIIa activity provided that Val-737 was conserved. The introduction of the 'active' *indica*-type *SSIIa* gene enabled the *japonica*-type cv. Kinmaze to synthesize *indica*-type amylopectin. The starch in the transformed *japonica* rice plants exhibited gelatinization-resistant properties that are characteristic of *indica*-rice starch. Transformed lines expressing different levels of the IR36 SSIIa protein produced a variety of starches with amylopectin chain-length distribution patterns that correlated well with their onset temperatures of gelatinization. The present study confirmed that the SSIIa activity determines the type of amylopectin structure of rice starch to be either the typical *indica*-type or *japonica*-type, by playing a specific role in the synthesis of the long B_1 chains by elongating short A and B_1 chains, notwithstanding the presence of functional two additional *SSII* genes, a single *SSI* gene, two *SSIII* genes, and two *SSIV* genes in rice plants.

Abbreviations: DP, degree of polymerization; DSC, differential scanning calorimetry; GBSS, granule-bound starch synthase; IPTG, isopropylthio-β-D-galactoside; SS, souluble starch synthase

Introduction

Starch synthase catalyzes the α-1,4-chain elongation reaction step in starch synthesis by transferring the glucose moiety of ADPglucose to the non-reducing end of α-1,4-chains. Plants have soluble starch synthases (SS) and granule-bound starch synthases (GBSS), which are responsible for the elongation of amylopectin chains and amylose, respectively. Biochemical and genetic studies indicate that plants have four types of SS, i.e., SSI, SSII, SSIII, and SSIV – some types having a number of multiple isoforms that varies among plant species. The complexity of plant SS is further indicated by recent studies on *Arabidopsis* and rice genomes. Rice plants have a total of eight forms of SS encoded by independent genes: one SSI; three SSII isoforms, namely SSIIa, SSIIb and SSIIc; two SSIII isoforms; and two SSIV isoforms. Almost all of the SS isoforms are expressed in the endosperm

(Hirose and Terao, 2004; Jiang *et al.*, 2004; Dian *et al.*, 2005). The reason(s) for the requirement of multiple types of SS in the synthesis of amylopectin remain to be elucidated (Smith *et al.*, 1997; Nakamura, 2002; Ball and Morell, 2003; James *et al.*, 2003) although several studies have established some specific functions of SSI (Commuri and Keeling, 2001) and the isoforms of SSII (Craig *et al.*, 1998; Umemoto *et al.*, 2002; Morell *et al.*, 2003; Zhang *et al.*, 2004) and SSIII (Gao *et al.*, 1998; Edwards *et al.*, 1999).

Amylopectin has a defined structure composed of tandem linked clusters (approximately 9–10 nm each in length) where linear α-1,4-glucan chains are regularly branched via α-1,6-glucosidic linkages, whereas the glycogens of bacteria and animals have a more randomly branched structure (Thompson, 2000). The distinct structure of amylopectin (referred to as a tandem-cluster structure) contributes to the crystalline organization of the starch granule (Gallant *et al.*, 1997). Variations of the cluster fine structure are responsible for variations in starch functional properties between species (e.g., corn starch vs. potato starch), tissues (e.g., storage starch vs. assimilatory starch) and genetic backgrounds (e.g., *japonica* rice starch vs. *indica* rice starch).

Rice varieties cultivated worldwide are largely classified into two groups designated as *japonica*-type and *indica*-type (Oka and Morishima, 1997). Rice starch quality, which sharply contrasts between *japonica* and *indica* rice varieties, greatly influences rice cooking and processing methods for food and industrial applications. The quality of starch in the two rice varieties are clearly distinguishable on the basis of the disintegration of starch granules in alkali or urea solution: starch granules of seed of *japonica* rice varieties are easily degraded in 1.3 M KOH or 4 M urea solution at room temperature, whereas those of *indica* varieties are resistant (Warth and Darabsett, 1914).

The dramatic difference in the degree of resistance to starch disintegration between the two varieties is attributed to the differences in the fine structure of amylopectin constituting the starch granule (Umemoto *et al.*, 1999; Nakamura *et al.*, 2002). The L-type amylopectin produced in many *indica* varieties differ from the S-type amylopectin synthesized in most *japonica* varieties in that the proportion of short amylopectin chains with degree of polymerization (DP) ≤10 is dramatically lower in the former. Our previous gene mapping analysis results prompted us to hypothesize that the *SSIIa* gene is responsible for the differences in amylopectin structure between the two rice varieties (Umemoto *et al.*, 2002), a possibility that was later supported by Zhenyu *et al.* (2003). If so, the relative contribution of SSIIa to the amylopectin structure should be remarkable because rice plants have a total of eight SS isoforms. We have set out to examine the mechanism of how SSIIa greatly impacts rice starch quality. In our recent paper (Umemoto *et al.*, 2004), we reported some variable SSIIa amino acids identified by SNP analysis in several lines of cultivated rice as candidates for causing the different levels of SSII activity but no evidence was provided. Also lacking are data on the effects of SSIIa activity levels on amylopectin structure and starch physicochemical properties. In the present investigation, we aimed to determine the amino acid(s) that are essential for SSIIa activity by independently overexpressing in *Escherichia coli* the wild-type *SSIIa* genes from a *japonica* and an *indica* rice cultivar, and all the possible chimeric *SSIIa* genes derived from shuffling of the wild-type *SSIIa* gene fragments, and by assaying and characterizing directly these SSIIa enzymes. In addition, to prove the physiological function of SSIIa in amylopectin biosynthesis, we introduced the *indica SSIIa* gene into *japonica* rice to examine whether the starch *japonica* S-type amylopectin of the host can be converted into an *indica* L-type amylopectin in the transformants and how the various levels of *indica* SSIIa protein affect the endosperm starch amylopectin structure and properties.

Materials and methods

Cloning and sequencing of cDNA encoding SSIIa of japonica *and indica rice cultivars*

The rice *japonica* cultivars Nipponbare and Kinmaze, and the *indica* cultivars IR36 and Kasalath were grown during the summer months in a paddy field. Seeds were harvested at the early- to mid-milking stage for the extraction of total RNAs from the developing endosperm using the method described by Nishi *et al.* (2001). cDNAs were synthesized from the total RNAs by reverse

transcription, used as templates for PCR for the *SSIIa* gene employing the sense primer 5′-TTAGGATCCTCGGGGCGACCAT-3′ and the antisense primer 5′-GGCGGACATGGTCTCTT-CAC-3′, and their nucleotide sequences were determined by a DNA sequencer (ABI 3000 Automated DNA Sequencer, Perkin–Elmer).

Cloning of the region encoding the 'mature' SSIIa for shuffling experiments

The rice SSIIa transit peptide was predicted to be encoded by the first 204 nucleotide bases (from the A nucleotide of the ATG start codon) based on the alignment of rice SSIIa deduced amino acid sequence with that of maize SSIIa, particularly with its putative transit peptide cleavage site VREA (*Harn et al.*, 1998), which corresponds to rice SSIIa VRRA (amino acid residue nos. 69–72). The rice SSIIa precursor was 'cleaved' between V and R to exclude the fist 68 amino acids, leaving a mature SSIIa protein of 742 amino acids (residues 69–810).

The region encoding the mature SSIIa (nucleotide bases 205–2430) in the *indica* cultivar IR36 and the *japonica* cultivars Nipponbare and Kinmaze were amplified by PCR using the sense primer 5′-CATAGACAAACGtCGCGCGGA-TGATGGGGAGAACG-3′ containing the *Psh*AI site paired with the antisense primer 5′-GGCGG-ACATGGTCTCTCACCATTGGTACTTGG-3′. The incorporation of the *Psh*A1 site in the sense primer, which required the replacement of the 5′ terminal base C to T(lower case), was needed for cloning into the expression vector pET 43.1b (Novagen). This base substitution, nevertheless, was silent. The single band PCR products were gel-purified and cloned in pGEM-T Easy (Promega Corp.) and sequenced. The correct inserts were excised by double digestion with *Psh*AI and *Sal*I and cloned into the same sites in pET43.1b.

Generation of SSIIa fragments

Four contiguous fragments of the coding region of the 'mature' SSIIa of the three rice cultivars were generated by restriction enzyme digestion of the *SSIIa*-pET43.1 constructs (Figure 1A). Thus, fragment 1 was generated with *Psh*A1 and *Eco*T22I; fragment 2 with *Eco*T22I and *Bsi*WI; fragment 3 with *Bsi*WI and *Xho*I; and fragment 4 with *Xho*I, the 3′ half of which contains portion of the MCS of pGEM-T Easy vector and that of pET43.1b. Fragments 1, 2, 3, and 4 are 970, 986, 143, and 273 bp, respectively, and contain the codons for Glu or Asp, Gly or Ser, Val or Met, and Leu or Phe, respectively. Corresponding fragments were shuffled to generate 16 constructs that cover all possible combinations. The resulting chimeric *SSIIa* genes in pET43.1b, which were verified for correctness by sequencing, were introduced into the expression host *E. coli* strain AD494 (DE3) pLysS (Novagen).

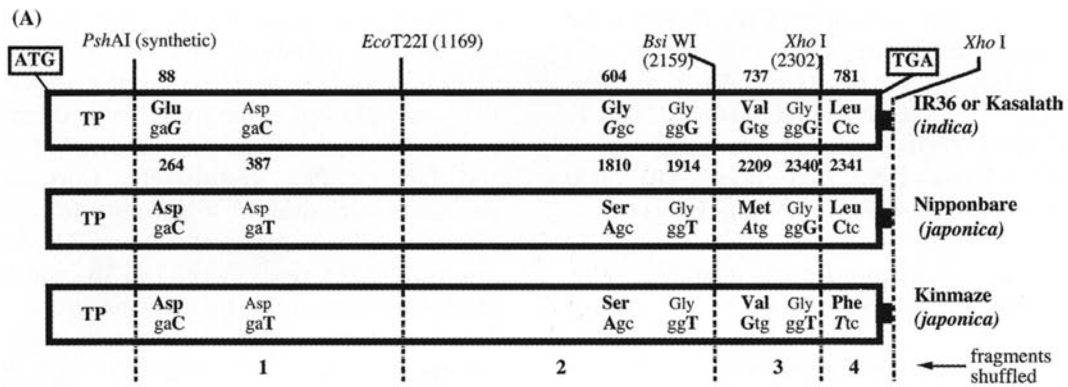
Induction and purification of SSIIa enzyme activity

The transformed *E. coli* AD494 cells were grown at 37 °C to OD_{600} of about 0.6 in LB medium containing 100 μg of carbenicillin and 34 μg of chloramphenicol per milliliter of the medium and divided into two portions. One portion was supplemented with IPTG to a final concentration of 1 mM, while the other served as the non-supplemented control. Both were cultured for an additional 2 h. The cells were collected by centrifugation at 20,000 × *g* for 2 min, and frozen at −80 °C until use. The frozen cells were thawed on ice, resuspended in phosphate-buffered saline (PBS), and disrupted by sonication on ice in an Ultrasonic Disruptor Model UD201 (Tomy Corp., Tokyo, Japan) set at a power output level of 3% and 30% duty for 10 bursts, centrifuged at 20,000 × *g* for 1 min at 4 °C, and the supernatants which were designated as the crude enzyme extracts were collected and frozen at −80 °C until use. The SSIIa protein was purified by sequential use of HitrapQ (Amersham Biosciences, Uppsala, Sweden) anion-exchange chromatography and TSK gel DEAE-5PW (Tosoh Corp., Tokyo) anion-exchange chromatography.

Measurements of SSIIa activities in vitro *assay and native-PAGE/activity staining assay*

Assays for the expressed SSIIa activity in *E. coli* crude enzyme extract and purified preparations, and SSIIa activity staining in native 7.5% polyacrylamide gel were performed as described by Nishi *et al.* (2001).

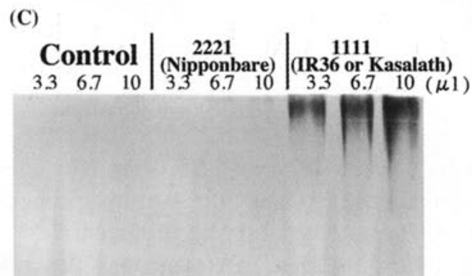
(A)



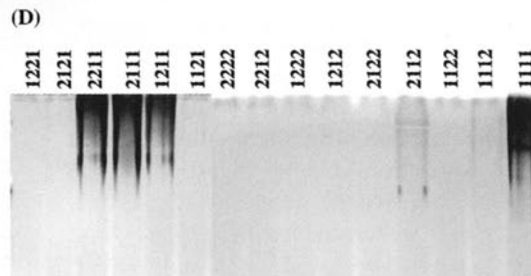
(B)

F1 (88)	F2 (604)	F3 (737)	F4 (781)	SSIIa Activity	Construct No.
Glu	Gly	Val	Leu	+	1111 (IR36 or Kasalath)
Glu	Gly	Met	Leu	−	1121
Glu	Gly	Val	Phe	±	1112
Glu	Gly	Met	Phe	−	1122
Asp	Gly	Val	Leu	+	2111
Asp	Gly	Met	Leu	−	2121
Asp	Gly	Val	Phe	±	2112
Asp	Gly	Met	Phe	−	2122
Glu	Ser	Val	Leu	+	1211
Glu	Ser	Met	Leu	−	1221
Glu	Ser	Val	Phe	−	1212
Glu	Ser	Met	Phe	−	1222
Asp	Ser	Val	Leu	+	2211
Asp	Ser	Met	Leu	−	2221 (Nipponbare)
Asp	Ser	Val	Phe	−	2212 (Kinmaze)
Asp	Ser	Met	Phe	−	2222

(C)



(D)



Characterization of purified SSIIa expressed in E. coli

To characterize the SSIIa proteins *in vitro*, their ability to synthesize specific polyglucans was examined. Purified *E. coli*-expressed IR36 or Kinmaze SSIIa protein was added into a reaction solution composed of 50 mM Bicine-NaOH (pH 7.4), 0.5 M citrate-NaOH (pH 7.4), 20 mM dithiothreitol, 2 mM ADPglucose, 0.9 mg amylopectin (prepared from amylose-free *japonica*-type rice cultivar Kinmaze) in a total volume of 0.45 ml. The enzymic reaction was run for 30 min at 30 °C, and terminated by placing the mixture in a boiling water bath for 2 min. Analysis of the chain-length distribution of polyglucans before and after the enzymic reaction was performed by capillary electrophoresis as described previously (Nakamura *et al.*, 2002).

Figure 1. *OsSSIIa* gene fragment shuffling. (A) Comparison of the *SSIIa* gene coding regions (represented by bars) in the *indica* cultivar IR36 and Kasalath, and the *japonica* cultivars Nipponbare and Kinmaze. Inside the bars are the variable amino acid residues (in bold, position given above the IR36 bar) and their respective codons, the italicized uppercase nucleotide bases being the ones that caused the amino acid changes (positions noted below the IR36 bar). The other variable nucleotides (in bold), which did not change the corresponding amino acids and their positions, are also shown. The four fragments of the putative mature *SSIIa* gene (excluding the transit peptide, TP) that were shuffled among the three cultivars (marked below the figure) were generated by digestion with the restriction enzymes given on top of the figure. Solid bars represent a portion of the fused multicloning site derived from the MCS of pGEM T-Easy and pET43b. The sizes of the fragments are not strictly drawn to scale. (B) Chimeric OsSSIIa constructs. Four contiguous fragments (F1, F2, F3, and F4) of the mature SSIIa coding regions of the *indica* cultivar IR36, and the *japonica* cultivars Nipponbare and Kinmaze were shuffled and expressed in *E. coli* AD494 using 1 mM IPTG as the inducer. Each of the shuffled gene fragments contains one variable amino acid residue, the position of which is indicated above the uppermost set of bars. The sizes of the fragments are not drawn to scale. The levels of SSIIa activities of the fusion proteins determined from clarified crude enzyme extract by native PAGE activity staining in non-denaturing acrylamide gel containing glycogen, as shown in (C) and (D), were classified into three types: +, normal SSIIa activity type (Type 1), including wild-type *indica*-cultivars IR36 and Kasalath; −, no or low activity type (Type 2 including wild-type *japonica*-cultivars Kinmaze and Nipponbare); ±, intermediately active type (Type 3). (C) and (D) Assay of SSIIa activity by native-PAGE activity staining method. Each lane contained 3.3, 6.7, and 10 μl in (C), as indicated, and 10 μl each in (D) of the crude enzyme extract of *E. coli*-expressed SSIIa.

Transformation of the japonica *rice and screening of transgenic lines*

The DNA construct containing the SSIIa cDNA from *indica* cultivar IR36 or *japonica* cultivar Nipponbare linked to the rice glutelin B_1 promoter (Takaiwa *et al.*, 1996) was introduced into *japonica* cultivar Kinmaze by *Agrobacterium tumefaciens* EHA105-mediated transformation (Hood *et al.*, 1993). Procedures for rice tissue culture, transformation and selection were as described previously (Kubo *et al.*, 2005).

Totals of 29 and 32 individual T_0 progeny lines were isolated from the IR36-SSIIa and Nipponbare-SSIIa transformants, respectively. Three to five T_1 seeds randomly chosen from each T_0 plant were independently analyzed for endosperm amylopectin chain-length distribution by capillary electrophoresis as described below. Ten randomly chosen T_1 seends of 12 T_0 IR36-SSIIa transformant lines were grown and their seeds (T_2) were examined for amylopectin chain-length distribution and starch gelatinization properties by differential scanning calorimetry. Five homozygous lines (#50-4, #63-1, #68-1, #73-8 and #78-2) were selected and their seeds (T_3 or T_4) were used for further biochemical studies.

Extraction of SSIIa protein from rice endosperm

Dry seeds of Kinmaze (50 mg), IR36, or transformant #78-2 was homogenized using plastic pestle in a microtube on ice in 250 μl of grinding solution (GS) containing 50 mM imidazole–HCl (pH 7.4), 8 mM $MgCl_2$, 50 mM 2-mercaptoethanol, and 12.5% glycerol and kept on ice for 10 min. The homogenate was centrifuged at 30,000 × *g* for 10 min at 4 °C and the pellet was washed twice with 250 μl of ice-cold GS. The supernatants were combined and used as the soluble protein (SP) fraction. The residual pellet was resuspended at 4 °C in 250 μl of an SDS solution containing 55 mM Tris–HCl (pH 6.8), 2.3% SDS, 5% 2-mercaptoethanol, and 10% glycerol and incubated for 10 min on ice. The homogenate was centrifuged at 30,000 × *g* for 10 min at 4 °C, and the precipitate was washed twice with 250 μl of ice-cold GS, followed by another wash with

100 μl of the SDS solution. The supernatants were combined and used as the loosely-bound protein (LBP) fraction. The resulting pellet (about 40 mg of starch granules) was washed with 250 μl of distilled water and twice with 2 ml of acetone, and dried *in vacuo*. The resulting starch granules (10 mg) were suspended in 1.8 ml of the SDS solution and boiled for 7 min. After cooling, the slurry was centrifuged at 40,000 × *g* for 15 min at 4 °C. The pellet was suspended in 200 μl of the SDS solution and centrifuged again. The combined supernatants were mixed with 2 ml of 30% trichloracetic acid and kept on ice overnight. The suspension was centrifuged at 40,000 × *g* for 15 min at 4 °C. The precipitate was washed twice with 1 ml of acetone, dried *in vacuo*, and solubilized with 30 μl of the SDS-PAGE solution containing 100 mM Tris–HCl (pH 6.8), 0.2% bromophenol blue, 10% SDS, 12% 2-mercaptoethanol, and 20% glycerol, and used as the tightly-bound protein (TBP) fraction.

Analysis of chain-length distribution of amylopectin and physicochemical properties of starch granules

Measurements of chain-length distribution of amylopectin by capillary electrophoresis and thermal properties of starch granules by differential scanning calorimetry were performed as described previously (Nakamura *et al.*, 2002). Analysis of pasting properties of starch granules by Rapid Visco Analyzer were carried out as described by Wong *et al.* (2003).

Accesion numbers

The cDNA sequences encoding the rice SSIIa were submitted to the DDBJ/NCBI/EMBL databases and were assigned the following accession numbers: Nipponbare, AB115915; Kinmaze, AB115916; IR36, AB115917; and Kasalath, AB115918.

Results

Comparison of the SSIIa nucleotide and amino acid sequences of japonica *and* indica *rice cultivars*

To reveal what causes the functional difference between the 'active' *SSIIa* gene in *indica* and 'inactive' *SSIIa* gene in *japonica* cultivars of rice, the nucleotide sequences of the cDNA of the *SSIIa* genes in the two indica-type rice cultivars (IR36 and Kasalath) that synthesize L-type amylopectin and two *japonica*-type cultivars (Nipponbare and Kinmaze) that synthesize S-type amylopectin were aligned using the Genetyx Analysis Software (Software Development Co. Ltd., Tokyo, Japan). Comparison of the four sequences showed that the length and the base composition of the *SSIIa* coding region were highly conserved. The eight peptide motifs conserved in all starch synthases of plants and prokaryotes as defined by Cao *et al.* (1999) were all present in the four deduced amino acid sequences of SSIIa. The *indica* cultivars had identical nucleotide sequences, whereas the *japonica* cultivars varied in three positions at their 3′ ends (Figure 1A). As a group, the *indica* cultivars differed from the *japonica* group at four positions: base no. 264 (G in *indica* vs. C in *japonica*); no. 387 (C vs. T); no. 1810 (G vs. A); and no. 1914 (G vs. T). Only the substitutions of bases no. 264 and no. 1810 resulted into changes in amino acid residues from Glu-88 to Asp in the former and from Gly-604 to Ser in the latter. At the 3′ end of the *SSIIa* gene, the three base substitutions in *japonica* cultivars were as follows: base no. 2209 (G) was changed to A in Nipponbare; and bases no. 2340–2341 (GC) were replaced by TT in Kinmaze. These base substitutions altered the two amino acid residues from Val-737 to Met in Nipponbare, and from Leu-781 to Phe in Kinmaze. The substitution of Val-737 to Met in Nipponbare could be critical in view of the fact that Val-737 is a component of Motif VIII in the catalytic region of SSIIa (Cao *et al.*, 1999). Altogether, the predicted SSIIa proteins differ only in four amino acid residues, one at the N terminal half and three at the C terminus. At least one of these amino acid residues is essential for SSIIa activity.

Shuffling of the SSIIa gene fragments

Despite the existence of only four variable amino acid residues in the predicted SSIIa proteins of the rice *japonica* and *indica* varieties, the SSIIa in the former appears to lack catalytic activity. This is indicated by the failure of *japonica* SSIIa to elongate short chains of DP ≤10 up to DP ≤24, which results into the formation of S-type amylopectin, as opposed to the L-type in *indica*

varieties that possess active SSIIa. It is likely that at least one of the four variable amino acids in the *indica* varieties is essential for SSIIa activity.

In order to identify the amino acid residue(s) critical for SSIIa activity, corresponding fragments of the SSIIa coding regions of the three cultivars were shuffled to generate chimeric *SSIIa* genes (Figure 1B), which were cloned in the expression vector pET43, expressed in *E. coli* AD 494, and the activity of SSIIa was measured by spectrophotometric and zymogram assay (Figure 1C and D). The native and chimeric SSIIa proteins could be largely classified into three types; the 'active' (*indica*-type, type 1) type, the 'inactive' (*japonica*-type, type 2) type, or 'intermediately active' type (type 3). SSIIa activity in the crude enzyme extract of the transformed *E. coli* cells appeared to be primarily determined by the Val-737 and Leu-781 residues at the C terminus. The presence of both amino acid residues conferred a type 1 activity regardless of the variations in the other regions of the SSIIa protein (constructs #1111 [IR36], 2111, 1211, and 2211). In these active chimeric SSIIa constructs, replacement of Val-737 with Met abolished SSIIa activity (construct #1111 [IR36, type 1] vs. 1121 [type 2], 2111 vs. 2121 [type 2], 1211 vs. 1221 [type 2], and 2211 vs. 2221 [Nipponbare, type 2], indicating that Val-737 is crucial for SSIIa activity. Surprisingly, replacement of Leu-781 with Phe only partially reduced SSIIa activity in chimeric genes having Gly-604 (construct #1111 [IR36, type 1] vs. 1112 [type 3], and 2111 [type 1] vs. 2112 [type 3]), but abolished it when Gly-604 was also replaced with Ser (construct #1211 [type 1] vs. 1212 [type 2] and 2211 [type 1] vs. 2212 [Kinmaze, type 2], implying that Gly-604 possibly interacts positively with Phe-781, although the sole replacement of Gly-604 with Ser did not result in loss of SSIIa activity when both Val-737 and Leu-781 were conserved. Replacing both Val-737 and Leu-781 with Met and Phe, respectively, rendered the SSIIa protein inactive. Because replacement of Glu-88 with Asp did not alter the activity of any constructs, Glu-88 apparently does not play an equally important role in determining SSIIa activity. These results suggest that Val-737 and Leu-781 are essential while Gly-604 plays some role in SSIIa catalytic activity when Leu-781 is replaced by Phe.

To prove that the functions of *indica* SSIIa and *japonica* SSIIa are different, direct determination of their enzyme activities was necessary. Since it is difficult to quantify the SSIIa activity in rice endosperm due to its extremely low expression level even in *indica* varieties, the SSIIa proteins from #1111 (wild-type IR36, type 1) and #1112 (Leu-781 replaced with Phe, type 3) were overexpressed in *E. coli*. Although the activities of type 1 and type 2 SSIIa could be easily measured by native-PAGE/activity staining method, no substantial activities of the crude *E. coli* extract expressing type 3 SSIIa protein were detected in crude enzyme extracts (Figure 1C and D). SSIIa activities were also measured by *in vitro* enzyme assay, but the levels of the type 3 SSIIa activities were similar to that of *E. coli* endogenous glycogen synthase activity (data not shown). To exclude the bacterial glycogen synthase activity from interfering with the expressed SSIIa activities, the SSIIa proteins were purified to almost homogeneity and subsequently used to quantify their enzymatic activities, if any. The purified SSIIa of the four selected constructs, i.e., #1111 (IR36), #2221 (Nipponbare), #2212 (Kinmaze), and #1112 (Leu-781 replaced with Phe) exhibited specific activities of 1.63, 0.13, and 0.15 μmol ADPglucose/min/mg protein for #1111, #2221 (Nipponbare) and #2212 (Kinmaze), respectively, and 0.72 μmol ADPglucose/min/mg protein for #1112, which was intermediate between *indica* (#1111) and *japonica* (#2221 or #2212) SSIIa (Figure 2).

Analysis of the structure of polyglucans synthesized by SSIIa from S-type amylopectin

To characterize the different SSIIa proteins, the SSIIa from *indica*-type (#1111) or *japonica*-type (Kinmaze, #2212) was incubated with S-type amylopectin, and the change in the chain-length distribution of the product was analyzed. Figure 3A and B shows that the *indica*-type SSIIa could convert S-type amylopectin to L-type amylopectin by elongating short chains of DP ≤ 11 to form chains of DP 13–25, although no change was observed in the proportion of long chains of DP ≥ 29 as indicated by the absence of any difference in the chain length distribution patterns between the product and substrate (S-type amylopectin), these patterns of difference being the same as those between IR36 L-type and Kinmaze S-type amylopectin (compare Figure 3B with Figure 5A,

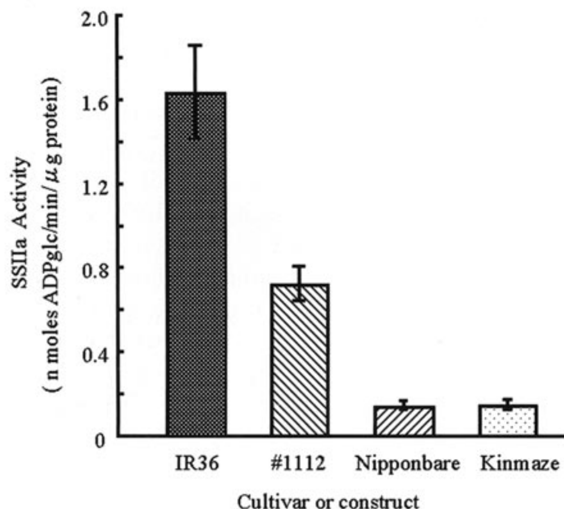


Figure 2. Specific activities (bars) of the HPLC-purified *E. coli* AD494-expressed SSIIa fusion proteins of the wild-type *indica* cultivar IR36, wild-type *japonica* cultivars Kinmaze and Nipponbare, and the chimeric line #1112. Bars and vertical lines on top of the bars are the means and standard deviations of 3 replicate measurements, respectively.

bottom panel). On the contrary, the chain profile for the polyglucan produced by incubation of S-type amylopectin with Kinmaze SSIIa was dramatically different from the above result, in that the proportion of short chains of DP 8–15 was increased while that of chains of DP ≥17 was decreased (Figure 3C). One possible explanation for this unexpected result might be that the *japonica*-type SSIIa elongated the endogenous glucan primers entrapped in the SSIIa protein itself. Whatever the reason is, however, the results clearly show that the *indica*-type SSIIa is distinct from *japonica*-type SSIIa not only in terms of specific activity, but also in the preference of substrate chains for elongation.

The amylopectin and starch in transgenic japonica *rice could have* indica*-type amylopectin and starch when the* indica *rice* SSIIa *gene was introduced*

The structural difference between the amylopectins of *indica* and *japonica* rice varieties is most clearly revealed by their chain-length profiles. The *japonica* S-type amylopectin has enriched short branch chains of DP ≤ 10 and depleted intermediate chains of 12 ≤ DP ≤ 24 as compared with the *indica* L-type amylopectin, whereas the proportion of cluster interconnecting long chains of DP ≥25 are comparable in the two varieties (Nakamura *et al.*, 2002; Umemoto *et al.*, 2002).

To examine whether the differences in the *SSIIa* gene is responsible for the structural differences between the L-type and S-type amylopectins, the *SSIIa* cDNA from the *indica* cultivar IR36 (having L-type amylopectin) or from the *japonica* cultivar Nipponbare (having S-type amylopectin) was introduced into the *japonica* cultivar Kinmaze (having S-type amylopectin). The cDNAs were individually cloned downstream of the endosperm-specific glutelin B_1 promoter to direct the expression of the introduced *SSIIa* gene only in the endosperm (Wu *et al.*, 1998), and the constructs were introduced independently into Kinmaze via *Agrobacterium tumefaciens*-mediated rice callus transformation.

Analysis of the chain-length distribution of amylopectin in T_1 seeds of transgenic lines confirmed that the S-type amylopectin structure of Kinmaze was converted into the L-type amylopectin in several lines transformed with the IR36-*SSIIa* gene (Figure 4A), but not in any lines transformed with the Nipponbare *SSIIa* gene (Figure 4B). The results suggest that the two *japonica* cultivars have defective *SSIIa* gene that could only synthesize the S-type amylopectin rather than the 'normal' L-type.

To examine the detailed mechanism for the complementation of L-type amylopectin biosynthesis by the 'normal' IR36 *SSIIa* gene, we selected five homozygous T_1 transformant lines (#50-4, #63-1, #68-1, #73-8 and #78-2) from 29 individual IR36-*SSIIa* (T_0) transformants. The seeds of these homozygous transformant (T_2) exhibited endosperm amylopectin chain-length profiles that differed from that of the wild-type cultivar Kinmaze. Phenotypic analyses were conducted on different seeds arbitrarily chosen from a pool of seeds harvested from a single homozygous T_3 or T_4 plant of each transformed line. Thus far, the specific phenotypes of each homozygous line were consistent among different plants and remained unchanged through succeeding generations (data not shown). Detailed phenotypic analyses were conducted using seeds from the homozygous line #78-2 (Figure 5). The pattern and the extent of differences in the branched chain profile between line #78-2 and Kinmaze were similar to those between IR36 and Kinmaze (Figure 5A) indicating that the chain-length profile of amylopectin of

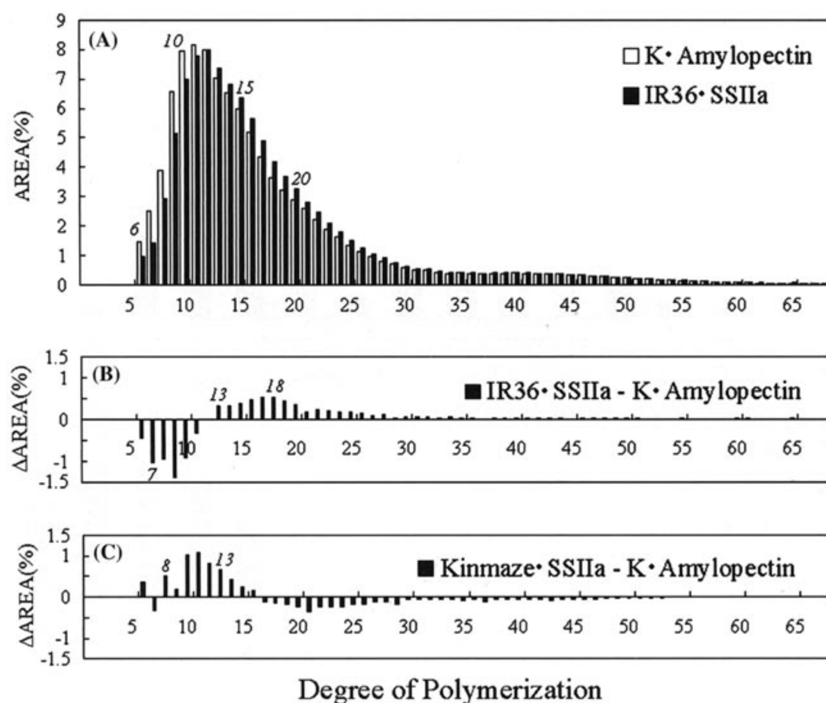


Figure 3. Changes in chain-length distribution of α-polyglucans resulting from the incubation of S-type amylopectin (prepared from amylose-free cv. Kinmaze) with purified SSIIa preparations. The enzymic reactions were performed as described in Materials and methods in a total volume of 0.45 ml. The protein amounts used were 7 and 40 μg for the *indica*-type rice cv. IR36 and the *japonica*-type rice cv. Kinmaze, respectively, to approximately equalize the catalytic activities of both enzymes in the reaction mixture because *japonica* SSIIa is comparatively much less active than that of *indica* SSIIa. The numbers shown in italic on some bars indicate the DP values of the corresponding bars. (A) Chain-length distribution of amylopectin prepared from *japonica*-type rice cultivar Kinmaze (open bars) and of polyglucans obtained after incubation of S-amylopectin with the purified IR36 SSIIa (closed bars). (B) and (C) Differences in polyglucan chain-lengths between the substrate (Kinmaze amylopectin) and the products of the enzymic reaction catalyzed by purified IR36 SSIIa (B) or Kinmaze SSIIa (C) preparation.

this line completely shifted from that of the S-type to the L-type. This alteration of amylopectin structure of line #78-2 was accompanied by starch gelatinization-resistant properties as indicated by the resistance of line #78-2 starch granules to 4 M urea solution like IR36 starch granules while those in Kinmaze were easily disintegrated (Figure 5B). The pasting properties of line #78-2 starch as measured by rapid-visco analyzer were distinct from Kinmaze (Figure 5C). The pasting temperature was increased from 69.5 °C of Kinmaze to 77.8 °C, the latter value being similar to that (75.5 °C) of IR36 (Figure 5C). However, other starch pasting parameters of line #78-2, such as peak viscosity, breakdown, and setback, were similar to those of Kinmaze starch rather than IR36 starch. These results suggest that the structural change in amylopectin induced by varied SSIIa activity determines some of the gelatinization and pasting properties of starch granules such as onset temperature for gelatinization, while other factors such as contents of amylose and amylopectin super-long chains of DP 85–180 (Takeda and Hizukuri, 1987), which are variety-specific and not controlled by SSIIa, account for the peak viscosity and setback, respectively, of the starch granules (Horibata *et al.*, 2004).

It is known that some forms of SS bind to the starch granules (Mu-Forster *et al.*, 1996). To characterize the SSIIa protein in rice endosperm, its binding capacity to the starch granule was examined. Almost all of the SSIIa proteins in IR36 were bound to starch granules, and a considerable fraction was so tightly-bound that it could not be dissociated from the starch granules by washing with 2.3% SDS solution at 4 °C (Figure 5D). In contrast, SSIIa protein in Kinmaze endosperm was only loosely-bound to starch granules (Figure 5D), suggesting that the *japonica*-type SSIIa protein has a severely reduced binding

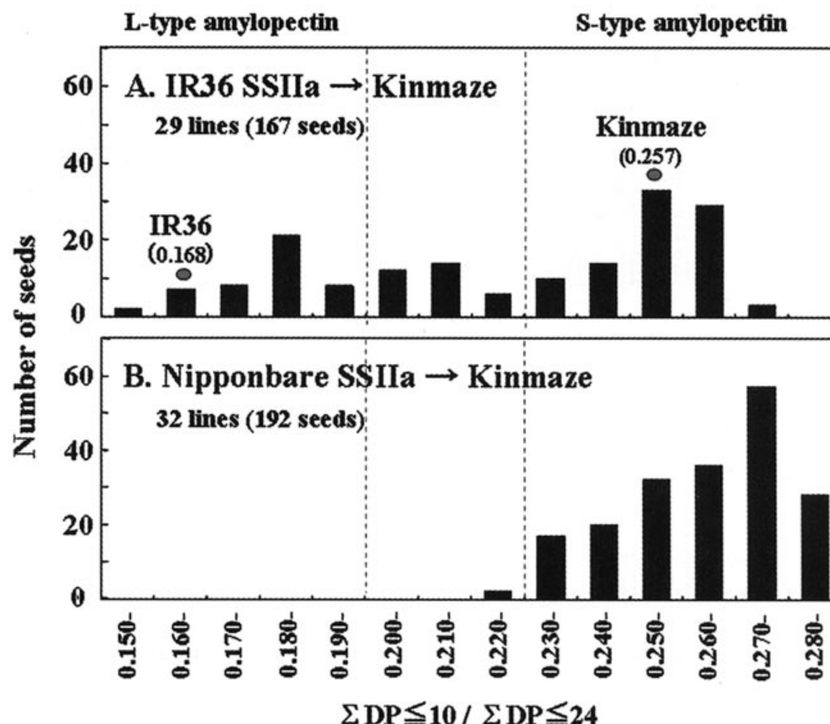
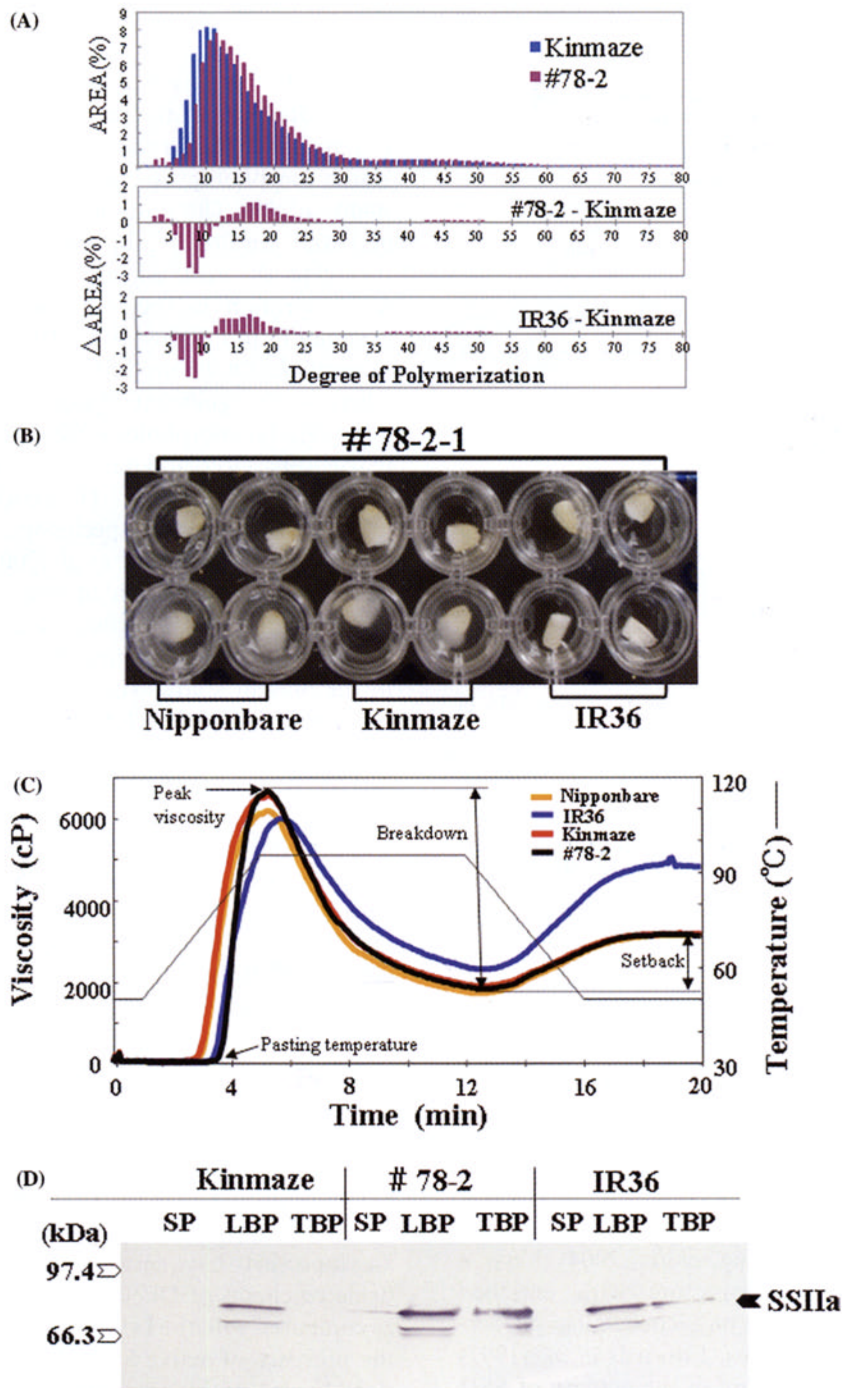


Figure 4. Variation in the structure of amylopectin in endosperm as measured by its α-1,4-chain-length distribution in transgenic *japonica*-rice cultivar Kinmaze. The transgenic lines were generated by introducing into Kinmaze the *SSIIa* cDNA from *indica*-rice variety IR36 (A) or *japonica*-rice variety Nipponbare (B). The histograms indicate the proportion of chains with DP ≤10 to that of chains with DP ≤24 in amylopectin obtained from a total of 167 seeds from 29 lines (independently chosen 3–5 seeds per line) of IR36-*SSIIa* transformants (A) and 192 seeds from 32 lines (3–5 seeds per line) of Nipponbare-*SSIIa* transformants (B).

capacity and probably lacks catalytic activity. In contrast, both loosely-bound and tightly-bound SSIIa proteins were observed in #78-2 as in the wild-type IR36 (Figure 5D). This and the above observations indicate that the expression of *indica*-type SSIIa in the Kinmaze transformant restored the functional SSIIa–starch granule interaction and the capacity of SSIIa to synthesize L-type amylopectin instead of S-type amylopectin.

The starch phenotypes of the five independent transformants with altered amylopectin chain-length were compared among each other and with IR36 and Kinmaze. Because the patterns of changes in the amylopectin chain-length distribution among all selected transformed lines examined were similar to those between IR36 and Kinmaze (Figure 6A) the proportion of short chains with DP ≤10 of the total cluster constituting chains with DP ≤24, as well as the amount of SSIIa protein tightly-bound to starch granules were compared with the onset temperature for gelatinization (T_0) of their starch granules. The

Figure 5. The effects of the introduction of the *indica*-rice cultivar IR36 *SSIIa* gene into the *japonica*-rice cultivar Kinmaze on the structure of amylopectin, gelatinization properties of starch granules, and the SSIIa protein level in the transformed homozygous line #78-2. (A) Comparison of α-1,4-chain-length distribution of endosperm amylopectin between transgenic line #78-2 or the *indica*-rice cultivar IR36 and its host *japonica*-rice cultivar Kinmaze. (B) Disintegration of starch granules of transgenic line #78-2, wild type *indica*-rice cultivar IR36, and *japonica*-rice cultivars Kinmaze and Nipponbare after incubation in 4 M urea solution at 30 °C for 18 h. (C) Pasting properties of starch granules of transgenic line #78-2, wild type *indica*-rice cultivar IR36, and *japonica*-rice cultivars Kinmaze and Nipponbare as measured by rapid-visco analyzer. (D) Western blot to quantify the relative amounts of soluble (SP), loosely starch-bound (LBP), and tightly starch-bound (TBP) SSIIa protein fractions from enzyme extracts of transgenic line #78-2, wild type *indica* cultivar IR36, and *japonica* cultivar Kinmaze. The amounts of proteins applied onto the gel were equivalent to 2.7% and 2.2% of the SP and LBP fractions, respectively. The amounts of applied protein were 13.9, 11.4 and 12.7% of the TBP fractions of Kinmaze, #78-2, and IR36, respectively. The protein blot was reacted with polyclonal antibodies raised against purified SSIIa from *japonica*-rice cultivar Nipponbare.



proportion of amylopectin short chains of DP ≤ 10 was inversely correlated with both the onset temperature for starch gelatinization (Figure 6B) and the amount of starch granule-tightly bound SSIIa protein (data not shown). In fact, the tightly-bound SSIIa protein amount was significantly higher in #78-2 than that in IR36 (Figure 5D). It appears that more active SSIIa (tightly granule-bound) generates amylopectin long chains that render the starch granules more difficult to gelatinize. Thus, some of the starch thermal properties are influenced by SSIIa activity.

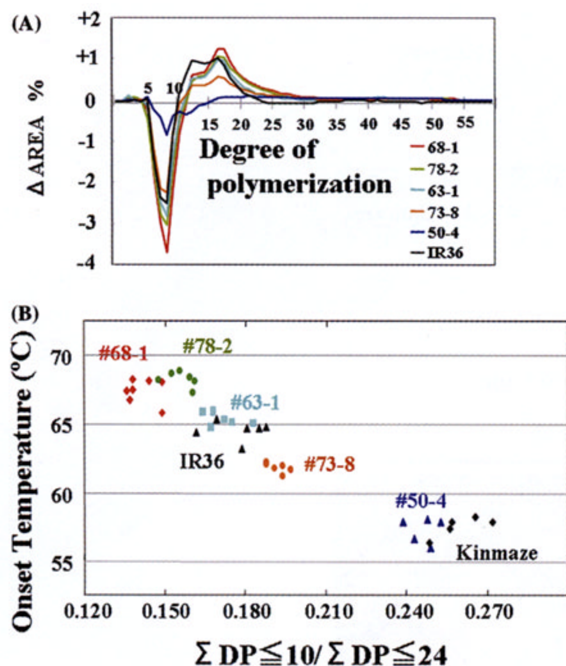


Figure 6. Relationship between the chain-length profile of amylopectin and starch gelatinization properties of seeds from various Kinmaze transgenic lines induced by introduction of the IR36-*SSIIa* cDNA. (A) Differences in α-1,4-chain-length distribution of endosperm amylopectin between the host *japonica* cultivar Kinmaze and IR36-*SSIIa* transformants or the *indica*-rice cultivar IR36. (B) Relationship between α-1,4-chain-length distribution of endosperm amylopectin and onset temperature of gelatinization of starch granules in IR36-*SSIIa* transformants and their host *japonica* cultivar Kinmaze, and the *indica* cultivar IR36. Each point represents data from 5 to 7 arbitrarily chosen seeds from each line.

Discussion

Several studies have been performed to dissect the function(s) of SSII or SSIIa. The SSII-deficient mutants from pea (Craig *et al.*, 1998), wheat (Yamamori *et al.*, 2000), barley (Morell *et al.*, 2003), and maize (Zhang *et al.*, 2004) have a modified amylopectin structure with enriched short chains, altering the physicochemical properties of the starch granules. Edwards *et al.* (1997) reported that the reduction in the activity of SSII in potato tuber by antisense technology causes the amylopectin to generate more chains of DP 8–12 and less chains of DP 15–25, but the proportion of chains longer than DP 25 is unaffected. The effect of inhibition of SSII activity on the amylopectin chain-length distribution pattern in potato is also quite similar to those in SSIIa mutants from rice (Umemoto *et al.*, 1999; Nakamura *et al.*, 2002), barley (Morell *et al.*, 2003), and maize (Zhang *et al.*, 2004). It is, however, noted that the phenotypic influences of the reduction of SSII (pea and potato) or SSIIa (cereals) on starch contents and starch morphology are markedly different among plant species. The mutation or inhibition of SSII activities results in shriveled seed morphology owing to the reduced amount of starch content and alteration in the size and shape of starch granules in pea (Craig *et al.*, 1998), wheat (Yamamori *et al.*, 2000), and barley (Morell *et al.*, 2003), whereas no significant changes in starch content and granular morphology are detected in seeds of maize (Shannon and Garwood, 1984), and rice (Umemoto *et al.*, 2004). The biochemical mechanism underlying these species-specific phenotypes is still unknown (Zhang *et al.*, 2004).

The specific function of rice SSIIa in amylopectin chain elongation was directly and conclusively proven by the *in vitro* experiment carried out in the present study. The *indica*-type SSIIa could synthesize L-type amylopectin when S-type amylopectin was incubated with the enzyme (Figure 3 A and B), whereas no such change occurred when the *japonica*-type enzyme was used (Figure 3C). The results clearly show that the active SSIIa from rice plays a specific role in the synthesis of the long cluster chains (B_1 chains) by elongating short A and B_1 chains, but not in the formation of cluster-connecting B_2 or longer chains.

The present investigation has proven that the level of active SSIIa form discriminates the fine structure of amylopectin between *japonica*-type and *indica*-type rice varieties having S-type and L-type amylopectin, respectively, by playing a distinct role in the elongation of short chains of DP ≤ 10 in the amylopectin cluster. The extremely low SSIIa activity (as in the *japonica*-type rice) caused the starch amylopectin to have enriched chains of DP 6–10 and depleted chains of DP 12–22 (S-type amylopectin) as compared with the L-type amylopectin formed in the presence of active SSIIa (as in the *indica*-type rice). In view of this apparently critical role of SSIIa in determining starch amylopectin structure, it is striking to note that the activity of SSIIa is relatively much lower than those of the two major SS isoforms in rice endosperm, namely SSI and SSIIIa, as reported in maize endosperm (Cao *et al.*, 1999), and that rice plants have eight SS isoforms including three SSII-type (Hirose and Terao, 2004).

There were four amino acid replacements between *indica* and *japonica* rice varieties (Figure 1A). The replacements of Glu-88 with Asp and Gly-604 with Ser were common between *indica* and *japonica* varieties, respectively, while those of Val-737 with Met (Nipponbare-type) and Leu-781 with Phe (Kinmaze-type) were variety-specific (Figure 1b). The *SSIIa* gene fragments shuffling experiments identified the amino acid residues crucial for rice SSIIa activity by showing that the replacement of either of two amino acids in *japonica* varieties (Val-737 to Met in Nipponbare, or from Leu-781 to Phe in Kinmaze) caused a marked reduction of SSIIa activity. However, it is particularly interesting that the SSIIa protein with Phe-781 exhibited a level of SSIIa activity that was intermediate between *indica* and *japonica* rice only when Ser-604 (*japonica*-type) was replaced by Gly (*indica*-type), whereas the sole replacement of Gly-604 with Ser had no inhibitory effect on the enzyme activity if both Val-737 and Leu-781 were conserved. The fact that the replacements of Leu-781 and Gly-604 in *indica*-type SSIIa to Phe-781 and Ser-604, respectively, in Kinmaze SSIIa resulted in the failure to synthesize L-type amylopectin from S-type amylopectin (Figure 3C) indicates that these changes in SSIIa protein not only reduce the SS catalytic activity, but also alter the chain preference for its elongation reaction and/or the affinity for glucan substrates.

Figure 6 shows that the extent of SSIIa-induced change in the fine structure of amylopectin was closely related with the onset temperature for starch gelatinization. Our previous report (Nakamura *et al.*, 2002) indicates that the type of amylopectin structure and starch thermal properties tend to segregate into either the typical *japonica*-type with no or little SSIIa activities or the typical *indica*-type with high SSIIa activities. The results of the present study show that in rice endosperm novel starch types with a variety of distinct functional properties can be engineered by modifying the SSIIa activity through intervarietal *SSIIa* gene transfer or shuffling the fragments of the *SSIIa* coding region. These strategies can generate SSIIa enzyme with varying activities that synthesize different amylopectins ranging from the L-type to the S-type.

The SSIIa-induced changes in the fine structure of amylopectin greatly influenced the physicochemical properties of the starch in rice endosperm such as thermal properties (Figure 6B) and pasting properties (Figure 5C). Recently, Horibata *et al.* (2004) reported elegant observations that the proportion of short chains of amylopectin cluster of DP 6–12 is inversely correlated with the peak temperature of gelatinization measured by DSC of rice starches, whereas amylose and amylopectin super-long chains contents are highly correlated with the peak viscosity and setback temperature, respectively, measured by rapid visco analyzer. These results seem to be consistent with the data in the present study (Figure 5C) in that the pasting temperature of starch in transformed line #78-2 was markedly increased by virtue of the expression of the introduced *indica*-type (IR-36) *SSIIa* in the host *japonica* rice cv. Kinmaze, whereas the peak viscosity, breakdown, and setback, were similar to those of Kinmaze starch rather than IR36 starch. The present results also support the view of Horibata *et al.* (2004) that the structural change in amylopectin induced by varied SSIIa activity determines some of the gelatinization properties and pasting properties of starch granules such as onset temperature for gelatinization, but not the peak viscosity and setback of the starch granules.

The present investigation demonstrated the molecular characterization of SSIIa of rice plants. The amino acid residues substituted in the putative C terminal region of *japonica* cultivars Nipponbare (Gly-604-Ser and Val-737-M) and Kinmaze (Gly-604-Ser and Leu-781-Phe) are different from those so far identified to be critical for substrate binding and catalysis in maize starch synthase, the only plant SS examined so far in terms of critical amino acid residues. In maize SSIIa, Arg-214, which is equivalent to Arg-354 of IR36, was implicated for catalysis (Imparl-Radosevich *et al.*, 1999), whereas Lys-193 (equivalent to Arg-333 of IR36) of the putative ADP-glucose binding site KTGGL motif was instead found to affect primer binding (Gao *et al.*, 2004). In maize SSIIb-2, Asp-21 and Glu-391 are apparently important for ADP-glucose binding, while Asp-139 is critical for enzyme activity (Nichols *et al.*, 2000). In IR36, these three maize SSIIb-2 residues are equivalent to Asp-339, Asp 457, and Glu-709, respectively.

IR36 SSIIa residue Val-737, which is the terminal component of conserved motif VIII (Cao *et al.*, 1999), corresponds to *E. coli* glycogen synthase (GS) Val-405 and *A. tumifasciens* GS Val-404, and is close to the critical region

369–374 in *E. coli* GS (Yep *et al.*, 2004) and 368–373 in *A. tumifasciens*. This Val residue appears to be conserved among plant and bacterial ADP-glucose-dependent glycosyltransferases and is conservatively replaced only by Ile in some plant granule-bound starch synthases (see Cao *et al.*, 1999). We observed that Kinmaze SSIIa was largely dissociated from the starch granules (Figure 5D). However, this cultivar had Glu-604-Ser and Leu-781-Phe substitutions, Val-737 being retained. Val-737 therefore may not be involved in substrate binding but most likely in catalysis as suggested by the abolition of SSIIa activity in construct #1112 in which only Val-737 was replaced (Figures 1 and 2). The inactivation of Nipponbare SSIIa may therefore be largely due to the non-conservative replacement of Val-737 by Met.

IR36 Gly-604 is equivalent to Asn-276 both in of *E. coli* and *A. tumifasciens* GS, and lies just before the strictly conserved Lys-277 residue, which in *E. coli* GS was proposed to be directly involved in catalysis (Furukawa *et al.*, 1994), based on the observation that in the Lys-277-Gln mutants, K_{cat} was reduced 140-fold relative to the wild-type. This observation, coupled with the information that the position of Lys-277 corresponds to those of the arginine clusters in the phosphorylation site of the GT3 glycosyltransferases family, prompted Buschiazzo *et al.* (2004) to postulate a 'long distance effect' of Lys-277 on catalysis, since Lys-277 of *A. tumifasciens* GS, like the arginine clusters of GT3, is spatially separated from the reaction center of the *E. coli* GS. In IR36 SSIIa, the equivalent residue Lys-605 might play a similar long distance role in catalysis, and could be influenced by the neighboring Gly-604, which when replaced by Ser as in *japonica* cultivars, is probably hindered from performing its function(s).

Further studies on rice SSIIa are obviously necessary in order to identify the role(s)/function(s) of Gly-604, Val-737, and Leu-781. Recently, Tetlow *et al.* (2004) presented direct evidences that in wheat, SSIIa, in addition to the SBE isoforms and unidentified proteins, is activated by phosphorylation. Since Gly-604 and Leu-781 are apparently not directly involved in binding and catalysis, they might play some roles in the phosphorylation-dependent activation of SSIIa.

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References

Ball, S.G. and Morell, M.K. 2003. From bacterial glycogen to starch: understanding the biogenesis of the plant starch granule Ann. Rev. Plant Biol. 54: 207–233.

Buschiazzo, A., Ugalde, J.E., Guerin, M.E., Shepard, W., Ugalde, R.A. and Alzari, P.M. 2004. Crystal structure of glycogen synthase: homologous enzymes catalyze glycogen synthesis and degradation EMBO J. 23: 3196–3205.

Cao, H., Imparl-Radosevich, J., Guan, H., Keeling, P.L., James, M.G. and Myers, A.M. 1999. Identification of the soluble starch synthase activities of maize endosperm Plant Physiol. 120: 205–215.

Commuri, P.D. and Keeling, P.L. 2001. Chain-length specificities of maize starch synthase I enzyme: studies of glucan affinity and catalytic properties Plant J. 25: 475–486.

Craig, J., Lloyd, J.R., Tomlinson, K., Barber, L., Edwards, A., Wang, T.L., Martin, C., Hedley, C.L. and Smith, A.M. 1998. Mutations in the gene encoding starch synthase II profoundly alter amylopectin structure in the pea embryos Plant Cell 10: 413–426.

Dian, W., Jiang, H. and Wu, P. 2005. Evolution and expression analysis of starch synthase III and IV J. Exp. Bot. 56: 623–632.

Edwards, A., Fulton, D.C., Hylton, C.M., Jobling, S.A., Gidley, M., Rössner, U., Martin, C. and Smith, A.M. 1999. A combined reduction in activity of starch synthase II and III of potato has novel effects on the starch of tubers Plant J. 17: 251–261.

Furukawa, K., Tagaya, M., Tanizawa, K. and Fukui, T. 1994. Identification of Lys277 at the active site of *Escherichia coli* glycogen synthase. Application of affinity labeling combined with site-directed mutagenesis J. Biol. Chem. 269: 868–871.

Gallant, D.J., Bouchet, B. and Baldwin, P.M. 1997. Microscopy of starch: evidence of a new level of granule organization Carbohydr. Polym. 32: 177–191.

Gao, Z., Keeling, P.L., Shibles, R. and Guan, H. 2004. Involvement of lysine-193 of the conserved "K-T-G-G" motif in the catalysis of maize starch synthase IIa Arch. Biochem. Biophys. 427: 1–7.

Gao, M., Wanat, J., Stinard, P.S., James, M.G. and Myers, A.M. 1998. Characterization of *dull1*, a maize gene coding for a novel starch synthase Plant Cell 10: 399–412.

Harn, C., Knight, M., Ramakrishnan, A., Guan, H., Keeling, P.L. and Wasserman, B.P. 1998. Isolation and characterization of the *zSSIIa* and *zSSIIb* starch synthase cDNA clones from maize endosperm Plant Mol. Biol. 37: 639–649.

Hirose, T. and Terao, T. 2004. A comprehensive expression analysis of the starch synthase gene family in rice (*Oryza sativa L.*) Planta 220: 9–16.

Hood, E.E., Gelvin, S.B., Melchers, L.S. and Hoekema, A. 1993. New Agrobacterium helper plasmids for gene transfer to plants Transgenic Res. 2: 208–218.

Horibata, T., Nakamoto, M., Fuwa, H. and Inouchi, N. 2004. Structural and physicochemical characteristics of endosperm starches of rice cultivars recently bred in Japan J. Appl. Glycosci. 51: 303–313.

Imparl-Radosevich, J.M., Keeling, P.L. and Guan, H. 1999. Essential arginine residues in maize starch synthase IIa are involved in both ADP-glucose and primer binding FEBS Lett. 457: 357–362.

James, M.G., Denyer, K. and Myers, A.M. 2003. Starch synthesis in the cereal endosperm Curr. Opin. Plant Biol. 6: 215–222.

Jiang, H., Dian, W., Liu, F. and Wu, P. 2004. Molecular cloning and expression analysis of three genes encoding starch synthase II in rice Planta 218: 1062–1070.

Jobling, S. 2004. Improving starch for food and industrial applications Curr. Opin. Plant Biol. 7: 210–218.

Kubo, A., Rahman, S., Utsumi, Y., Li, Z., Mukai, Y., Yamamoto, M., Ugaki, M., Harada, K., Satoh, H., Konik-Rose, C., Morell, M. and Nakamura, Y. 2005. Complementation of *sugary-1* phenotype in rice endosperm with the wheat *Isoamylase1* gene supports a direct role for isoamylase1 in amylopectin biosynthesis Plant Physiol. 137: 43–56.

Morell, M.K., Kosar-Hashemi, B., Cmiel, M., Samuel, M.S., Chandler, P., Rahman, S., Buleon, A., Batey, I.L. and Li, Z. 2003. Barley *sex6* mutants lack starch synthase IIa activity and contain a starch with novel properties Plant J. 34: 173–185.

Mu-Forster, C., Huang, R., Powers, J.R., Harriman, R.W., Knight, M., Singletary, G.W., Keeling, P.L. and Wasserman, B. 1996. Physical association of starch biosyntheitic enzymes with starch granules of maize endosperm Plant Physiol. 111: 821–829.

Nakamura, Y. 2002. Towards a better understanding of the metabolic system for amylopectin biosynthesis in plants: rice endosperm as a model tissue Plant Cell Physiol. 43: 718–725.

Nakamura, Y., Sakurai, A., Inaba, Y., Kimura, K., Iwasawa, N. and Nagamine, T. 2002. The fine structure of amylopectin in endosperm from Asian cultivated rice can be largely classified into two classes Starch 54: 117–131.

Nichols, D.J., Keeling, P., Spalding, M. and Guan, H. 2000. Involvement of conserved aspartate and glutamate residues in the catalysis and substrate binding of maize starch synthase Biochemistry 39: 7820–7825.

Nishi, A., Nakamura, Y., Tanaka, N. and Satoh, H. 2001. Biochemical and genetic analysis of the effects of *amylose-extender* mutation in rice endosperm Plant Physiol. 127: 459–472.

Oka, H. and Morishima, H. 1997. Wild and cultivated rice. In: T. Matsuo, Y. Futsuhara, F. Kikuchi and H. Yamaguchi (Eds.), Science of the Rice Plants. Genetics, Nobunkyo, Tokyo, 88–111 pp.

Shannon, J.C. and Garwood, D.L. 1984. In: Starch: Chemistry and Technology, Academic Press, Orlando, FL, 25–86 pp.

Smith, A.M., Denyer, K. and Martin, C. 1997. The synthesis of the starch granules Ann. Rev. Plant Physiol. Plant Mol. Biol. 48: 67–87.

Takaiwa, F., Yamanouchi, U., Yoshihara, T., Washida, H., Tanabe, F., Kato, A. and Yamada, K. 1996. Characterization of common cis-regulatory elements responsible for the endosperm-specific expression of members of the rice glutelin Plant Mol. Biol. 30: 1207–1221.

Takeda, Y. and Hizukuri, S. 1987. Structures of rice amylopectins with low and high affinities for iodine Carbohydr. Res. 168: 79–88.

Tetlow, I.J., Wait, R., Lu, Z., Akkasaeng, R., Bowsher, C.G., Esposito, S., Kosar-Hashemi, B., Morell, M.K. and Emes, M.J. 2004. Protein phosphorylation in amyloplasts regulates starch branching enzyme activity and protein–protein interactions Plant Cell 16: 694–708.

Thompson, D.B. 2000. On the non-random nature of amylopectin branching Carbohydr. Polym. 43: 223–239.

Umemoto, T., Aoki, N., Lin, H., Nakamura, Y., Inouchi, N., Sato, Y., Yano, M., Hirabayashi, H. and Maruyama, S. 2004. Natural variation in rice *starch synthase IIa* affects enzyme and starch properties Functional Plant Biol. 31: 671–684.

Umemoto, T., Nakamura, Y., Satoh, H. and Terashima, K. 1999. Differences in amylopectin structure between two rice varieties in relation to the effects of temperature during growth Starch 51: 58–62.

Umemoto, T., Yano, M., Satoh, H., Shomura, A. and Nakamura, Y. 2002. Mapping of a gene responsible for the difference in amylopectin structure between *japonica*-type and *indica*-type rice varieties Theor. Appl. Genet. 104: 1–8.

Warth, F.J. and Darabsett, D.B. 1914. Disintegration of rice grains by means of alkari Bull. Agric. Res. Inst. Pasa 38: 1–9.

Wong, K.S., Kubo, A., Jane, J.L., Harada, K., Satoh, H. and Nakamura, Y. 2003. Structures and properties of amylopectin and phytoglycogen in the endosperm of *sugary-1* mutant of rice J. Cereal Sci. 37: 139–149.

Wu, C.Y., Suzuki, A., Washida, H. and Takaiwa, F. 1998. The GCN4 motif in a rice glutelin gene is essential for endosperm-specific gene expression and is activated by Opaque-2 in transgenic rice plants Plant J. 14: 673–683.

Yamamori, M., Fujita, S., Hayakawa, K., Matsuki, J. and Yasui, T. 2000. Genetic elimination of a starch granule protein, SGP-1, of wheat generates an altered starch with apparent high amylase Theor. Appl. Genet. 101: 21–29.

Yep, A., Ballicora, M.A., Sivak, M.N. and Preiss, J. 2004. Identification and characterization of a critical region in the glycogen synthase from *Escherichia coli* J. Biol. Chem. 279: 8359–8367.

Zhang, X., Colleoni, C., Ratushna, V., Sirghie-Colleoni, M., James, M.G. and Myers, A.M. 2004. Molecular characterization demonstrates that the *Zea mays* gene *sugary2* codes for the starch synthase isoform SSIIa Plant Mol. Biol. 54: 865–879.

Zhenyu, G., Dali, Z., Xia, C., Yihua, Z., Meixian, Y., Danian, H., Jiayang, L. and Qian, Q. 2003. Map-based cloning of the *ALK* gene, which controls the gelatinization temperature of rice Sci. China 46: 661–668.