Starch-Branching Enzyme I-Deficient Mutation Specifically Affects the Structure and Properties of Starch in Rice Endosperm[1]

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We have isolated a starch mutant that was deficient in starch-branching enzyme I (BEI) from the endosperm mutant stocks of rice (*Oryza sativa*) induced by the treatment of fertilized egg cells with *N*-methyl-*N*-nitrosourea. The deficiency of BEI in this mutant was controlled by a single recessive gene, tentatively designated as *starch-branching enzyme mutant 1* (*sbe1*). The mutant endosperm exhibited the normal phenotype and contained the same amount of starch as the wild type. However, the mutation apparently altered the fine structure of amylopectin. The mutant amylopectin was characterized by significant decrease in both long chains with degree of polymerization (DP) ≥ 37 and short chains with DP 12 to 21, marked increase in short chains with DP ≤ 10 (A chains), and slight increase in intermediate chains with DP 24 to 34, suggesting that BEI specifically synthesizes B_1 and B_{2-3} chains. The endosperm starch from the *sbe1* mutant had a lower onset concentration for urea gelatinization and a lower onset temperature for thermo-gelatinization compared with the wild type, indicating that the genetic modification of amylopectin fine structure is responsible for changes in physicochemical properties of *sbe1* starch.

The current concept for starch biosynthesis in higher plants is that amylopectin, the major component of starch, is synthesized by concerted actions of ADP-Glc pyrophosphorylase (AGPase), soluble starch synthase (SS), starch-branching enzyme (BE), and starch-debranching enzyme (Smith et al., 1997). Because BE can only introduce α-1,6 glucan branches in the α-polyglucans, the enzyme is considered to influence the fine structure of amylopectin. Plant BEs might play specific roles in determining amylopectin tandem-cluster structure where α-1,6 glucan branches are localized at the basal portion of the cluster component, of which the length is relatively constant among various plant species (Jenkins et al., 1993).

All higher plants studied so far possess two classes of BE. They are referred to as BEI and BEII in maize (*Zea mays*; Boyer and Preiss, 1978; Fisher and Boyer, 1983; Guan and Preiss, 1993; Guan et al., 1997), rice (*Oryza sativa*; Nakamura et al., 1992; Mizuno et al., 1993), wheat (*Triticum aestivum*; Morell et al., 1997), and barley (*Hordeum vulgare*; Sun et al., 1997), and as B-type and A-type in pea (*Pisum sativum*; Burton et al., 1995; Martin and Smith, 1995), kidney bean (*Phaseolus vulgaris*; Hamada et al., 2001), and potato (*Solanum tuberosum*; Larsson et al., 1996, 1998). They can be distinguished from each other based on their distinct biochemical and physico-chemical properties, e.g. kinetic parameters for various α-polyglucans (Guan and Preiss, 1993; Takeda et al., 1993; Guan et al., 1997; Morell et al., 1997), chromatographic behaviors in anion-exchange and hydrophobic chromatograms (Nakamura et al., 1992), reactivities to chemicals such as cyclodextrins (Vikso-Nielsen and Blennow, 1998) and phosphorylated compounds (Morell et al., 1997), temperature responses (Takeda et al., 1993), association with starch granules (Mu-Forster et al., 1996), and their expression modes during plant development (Mizuno et al., 1993; Burton et al., 1995; Morell et al., 1997). Several investigations have shown that the genes encoding BEI and BEII belong to distinct gene families (Burton et al., 1995; Martin and Smith, 1995).

Biochemical observations with purified BEI and BEII isoforms from maize endosperm indicate that BEI preferentially branches amylose-type fewer branched polyglucans, whereas BEII has a higher capacity for branching amylopectin-type highly branched α-glucans (Guan and Preiss, 1993; Takeda et al., 1993; Guan et al., 1997). These data strongly suggest that BEI and BEII play distinct roles in the synthesis of amylopectin molecules (Nakamura, 2002; Nakamura et al., 2003). This idea is further supported by biochemical and genetic analyses of BEIIb-deficient mutants, such as the *amylose-extender* (*ae*) mutants of maize (Stinard et al., 1993) and rice

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(Mizuno et al., 1993) endosperms and the *rugosus* mutant of pea embryo (Bhattacharyya et al., 1990). The average chain length of *ae* amylopectin in maize endosperm is significantly longer than that of the wild-type amylopectin (Baba and Arai, 1984; Kasemsuwan et al., 1995). Biochemical and physicochemical analyses of *ae* mutant in rice demonstrated that BEIIb is involved in the transfer of short chains with degree of polymerization (DP) $\leq$ 17, suggesting that BEIIb plays an important role in the formation of A chains of amylopectin (Nishi et al., 2001).

The general concept that BEI and BEII play distinct roles in amylopectin biosynthesis in plant tissues has been drawn mainly from in vitro experiments with purified enzymes. To assess the physiological roles of both BEI and BEII, in vivo experiments, such as analysis of mutants and transgenic plants where BEI is specifically lacking or overexpressed are important. Up to now, however, attempts to identify the specific role of BEI by using BEI-deficient mutants have not been successful. Recently, Blauth et al. (2002) isolated for the first time a BEI-lacking mutant from maize. However, they found that the structure of starch in the endosperm of the mutant is not altered. This may be due to the low preference of BEI isoform(s) for α-glucans with different chain length as compared with BEII isoform(s) or the presence of multiple BEI isoforms that can complement each other in maize endosperm.

We have generated various kinds of mutants for rice endosperm starch by *N*-methyl-*N*-nitrosourea (MNU) treatment of fertilized egg cells (Satoh and Omura, 1981; Satoh, 1985; Satoh et al., 2003). Using SDS-PAGE to screen for the BEI-deficient mutants, we detected a mutant line lacking the BEI protein in its endosperm. In this paper, we report for the first time that the absence of BEI specifically modifies the structure of amylopectin and the physicochemical properties of starch in rice endosperm. The specific role of BEI in determining the amylopectin fine structure in rice endosperm is also discussed.

RESULTS

We have induced various kinds of mutations for endosperm traits by the treatment of fertilized egg cells of the *japonica* type rice (*O. sativa* cv Kinmaze and Taichung 65 [T65]) with MNU, a chemical mutagen (Satoh and Omura, 1981; Satoh, 1985; Satoh et al., 2003). More than 1,500 endosperm mutant lines used in this study are deposited at the Plant Genetic Resources Laboratory of Faculty of Agriculture in Kyushu University, Japan. Because these mutant lines were produced by the MNU treatment of fertilized egg cells within one cell cycle, the M_1 plants do not possess the chimera sector (Satoh and Omura, 1979). It is considered that these mutations cover the whole rice genome, and most of the mutants were induced by single-gene mutation. Therefore, the use of the mutant library is of great advantage in selecting biochemical or morphological traits caused by lesion of individual genes coding for one of the starch-metabolizing enzymes in rice endosperm. In the present study, we tried to select a mutant that lacks BEI in its developing endosperm.

Screening and Isolation of BEI-Deficient Mutant Lines

In this article, the terminology of three BE isoforms in rice endosperm is the same as described previously (Nishi et al., 2001). When the crude protein extract of the wild-type endosperm was subjected to SDS-PAGE analysis, only a single band corresponding to the 83-kD Coomassie-staining band was cross-reacted with the polyclonal antibodies raised against purified BEI from developing rice endosperm. Among 1,041 mutant lines examined, only a single line, EM557 derived from rice cv T65 lacked the BEI band (Fig. 1A), although a lot of lines classified as the *floury2* (*flo2*) type mutation were characterized by the decrease in the amount of BEI, as reported previously (Kawasaki et al., 1996). No BEI activity was found in the soluble enzyme extract of developing mutant endosperm (Fig. 1B). The loss of the BEI protein in EM 557 was also examined by immunoblot detection (Fig. 1C). The transcript of the *BEI* gene was also missing in EM 557 (Fig. 1D), indicating that the *sbe1*

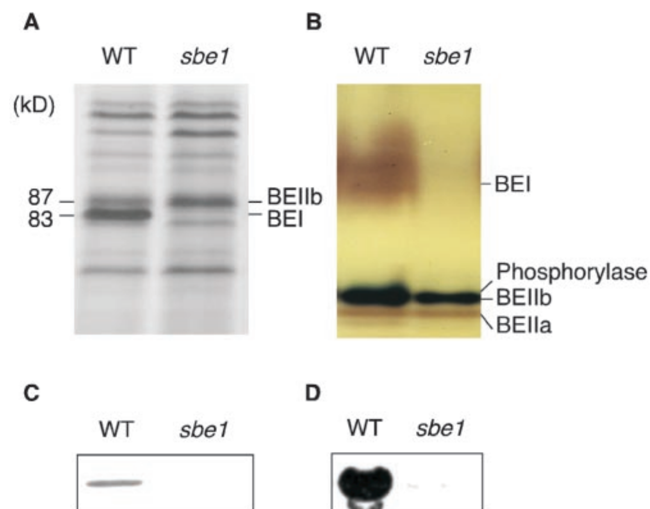

Figure 1. Effects of *sbe1* (BEI-deficient) mutation on the expression of BEI in endosperm. A, SDS-PAGE profile of the crude protein extract of rice endosperm in mature rice kernels. B, Native-PAGE/activity staining of BEs in developing seed of rice. The migration and identification of each band corresponding to three BE isoforms (BEI, BEIIa, and BEIIb) and phosphorylase were according to our previous report (Yamanouchi and Nakamura, 1992). The volumes of crude enzyme extracts applied were 0.67 μL. C, Western-blot analysis of BEI in mature rice kernels. The immunoblot was developed with antiserum raised against BEI from rice endosperm (Nakamura et al., 1992) at a dilution of 1/1000. D, Northern-blot analysis of BEI transcripts in rice endosperm. Total RNA from developing grains was blotted and probed with the specific RNA probe, EST clone EST#17(EC0727).

mutation inhibits the expression of *Sbe1* gene at the transcriptional level.

Genetic Analysis of Endosperm Mutant EM557 Lacking the BEI Protein

The mature kernel of the original mutant line EM557 exhibited a floury phenotype. In F_2 seeds derived from a cross between EM557 and the wild-type variety rice cv T65, both floury endosperm and BEI deficiency segregated to fit the expected ratios of 3:1 (Table I). These results indicate that both characters are controlled by respective single genes. Interestingly, however, no correlation was observed between the floury phenotype and BEI deficiency, i.e. the segregation mode of both characters fitted well the expected ratio of 9:3:3:1 (data not shown). This result indicates that both characters are controlled by different single genes and are inherited independently. In F_3 derived from the cross between rice cv T65 and EM557, BEI-deficient segregants without the floury character were isolated. They were crossed again to rice cv T65 to generate plants homozygous for BEI deficiency. The mutant lines from the crosses, which were named EM557S, were used as a material in the following experiments.

EM557S was crossed with the following eight non-allelic starch biosynthesis mutant lines: *ae* (EM10), *waxy* (*wx*; EM21), *flo1* (EM17), *flo2* (EM36), *sugary1* (*sug1*; EM41), *sug2* (EM75), *shrunken1s* (*shr1s*; EM20), and *shr2* (EM22). The phenotypes of endosperm in the F_1 seeds and the segregation modes in the F_2 were examined. The phenotypes of F_1 seeds from crosses between EM557S and other mutants had a normal appearance. The segregation modes of BEI deficiency and individual mutant characters in the F_2 progenies of the above crosses fitted the expected ratio of 9:3:3:1 (data not shown). These results confirm that the BEI deficiency gene is located apart from the *Ae*, *Wx*, *Flo1*, *Flo2*, *Sug1*, *Sug2*, *Shr1*, and *Shr2* alleles. On the basis of the above observations, we tentatively designated the gene for BEI deficiency in EM557S as *starch-branching enzyme mutant 1* (*sbe1*).

Nakamura et al. (1994) reported that the rice gene encoding BEI (*OsBEI*) is located on the long arm of chromosome 6. Trisomic and linkage analyses confirmed that the gene for BEI deficiency is located on the long arm of chromosome 6 (Table II). RFLP for *OsBEI* gene was detected between an *indica* rice cv Kasalath and EM557S by using expressed sequence tag (EST) clone EST#17 (EC 0727; Nakamura et al., 1994) as a probe. RFLP analysis of the F_2 population derived from the cross between rice cv Kasalath and EM557S showed that BEI deficiency cosegregated completely with an RFLP of EM557S for BEI (Table III), suggesting strongly that *sbe1* mutation is caused by the lesion of the gene encoding BEI in rice endosperm.

Table I. *Genetic behavior of sbe1 in rice*

Segregation mode of BEI deficiency (*sbe1* mutation type) in F_2 progenies of a cross between T65 (+) and EM557S (*sbe1*)

Cross Combination	F_1	Segregation in F_2 Seeds		Total	χ^2 (3:1)
		+	*sbe1*		
T65 × EM557 (+) (*sbe1*)	+	58	22	80	0.27

Effects of *sbe1* Mutation on Starch Accumulation in Endosperm and Grain Morphology

Figure 2 illustrates the morphology of whole kernel of the BEI-lacking mutant line BMF71 (EM557S). The mature kernel phenotype was normal like that of the wild-type rice cv T65, not only in appearance but also in the size and weight of the grain, whereas BEIIb-deficient *ae* mutant line EM529 had a significantly smaller kernel with floury appearance (Fig. 2; Table IV). Thus, it was impossible to distinguish EM557S from the wild type in kernel phenotype. Homozygous *sbe1* offspring (EM557S) did not exhibit any differences from rice cv T65 in terms of growth habits, such as morphology and heading date.

To obtain the amylose-free *sbe1* starch, EM557S (*sbe1 sbe1*/*Wx Wx*) was crossed with the amylose-free *wx* mutant line EM583 (*Sbe1 Sbe1*/*wx wx*), which was also induced by MNU treatment of rice cv T65, and the double-deficient mutant lines having the genotype *sbe1 sbe1*/*wx wx* were isolated from the F_3 population. The grain of amylose-free *sbe1* mutant lines also did not exhibit any difference in phenotype from those of the *wx*-counterpart (data not shown).

Effects of *sbe1* Mutation on the Levels of BEIIa, BEIIb, and the Other Amylopectin-Synthesizing Enzymes

To examine the pleiotropic effects of the *sbe1* mutation on the other BE isoforms, i.e. BEIIa and BEIIb, EM557S was crossed reciprocally with the original rice cv T65, and the expression levels of BEI, BEIIa, and BEIIb proteins in F_1 seeds were investigated (Fig. 3). The gene dosage effect of *Sbe1* gene on BEI protein was clearly evident among genotypes. Figure 3, A and B, shows that the BEI protein band was reduced with the decrease in the normal *Sbe1* gene dosage. The BEIIb protein band increased slightly (113% ± 9% of the wild type) when the *Sbe1* dosage was null (Fig. 3B), whereas the BEIIa protein band was present at the same level as in the wild type (data not shown).

The activities of SS and AGPase were similar between the mutant and the wild type (Table V). Zymogram analyses showed no significant differences in the activities of SS isoforms, isoamylase and pullulanase (data not shown). Therefore, it is likely that the mutation of the gene encoding BEI has no pleiotropic effect on other starch metabolizing enzymes.

Table II. *Genetic behavior of sbe1 in rice*

Trisomic segregation of BEI deficiency (*sbe1* mutation type) in F_2 plants from a trisomic F_1 of a cross between Triplo6 and EM557S (*sbe1*)

Cross Combination	F_1	F_2			Total	χ^2		
	Plant Type	Plant Type	+	*sbe1*		(3:1)	(8:1)	(44:1)
Triplo6 × EM557	T[a]	T	33	1	34			0.08
(*sbe1*)		D[b]	82	12	94		0.26	
	D		59	21	80	0.07		

[a] Trisomic plant. [b] Disomic plant.

Effect of *sbe1* Mutation on the Fine Structure of Amylopectin in Mature Endosperm

In an attempt to elucidate the structural changes of amylopectin in the endosperm of *sbe1* mutant, starch granules from mature seeds of the wild type, the *sbe1* mutant, the *wx* mutant, and the *sbe1/wx* double mutant were treated with isoamylase from *Pseudomonas amyloderamosa*, and then the chain length distribution was examined by 8-amino-1,3,6-pyrenetrisulfonic acid (APTS)-labeled starch using a high-resolution capillary electrophoresis and a laser-induced fluorescent detector. Because APTS is attached at the reducing end of the α-1,4-glucan chain, the peak area of each glucan with different DP can be compared on molar basis. In this analysis, multiple homozygous mutant lines independently chosen were examined to evaluate the effects of the *sbe1* mutation on amylopectin structure. Figure 4A presents that as compared with wild-type rice cv T65 amylopectin, the proportions of chains with DP $\geq$ 37 and 12 $\leq$ DP $\leq$ 21 of the *sbe1* mutant amylopectin were depressed, whereas those of chains with DP $\leq$ 10 as well as chains with 24 $\leq$ DP $\leq$ 34 were elevated. Although the extent of difference in the chain length distribution between the *sbe1* mutant and wild-type amylopectins was not marked, the same trend was detected in four homozygous mutant lines (Fig. 4A). The same pattern of changes in the amylopectin chain profiles was found in the two amylose-free *sbe1/wx* mutant lines (Fig. 4B). The results indicate that the loss of BEI results in alteration of amylopectin structure in rice endosperm.

In contrast, the chain distribution of *ae* amylopectin was distinctly dissimilar to that of *sbe1* amylopectin (Fig. 4C). In the *ae* amylopectin, the short chains of DP $\leq$ 13 markedly reduced, with the greatest decrease in chains with DP 7 to 12, whereas the long chains of DP $\geq$ 39 and 15 $\leq$ DP $\leq$ 34 increased. It is noted that the mode of the difference of amylopectin chain profiles between the *ae* mutant and wild type was distinct from that between *sbe1* mutant and wild type (Fig. 4, compare C with A). These results show that BEI and BEIIb play specific roles in amylopectin biosynthesis in rice endosperm.

Table III. *Genetic behavior of sbe1 in rice*

Cosegregation of BEI deficiency (*sbe1* mutation type) and *OsBEI* gene in F_3 from homozygous BEI deficient F_{2s} (*sbe1/sbe1*) derived from a cross between an *indica* rice cv Kasalath and EM557S.

	F_3 from F_{2s} of Homozygous BEI		
OsBEI	KK[a]	KJ[b]	JJ[c]
Number of plants	0	0	78

[a] RFLP of Kasalath type. [b] RFLP of heterozygous Kasalath and EM5575. [c] RFLP of EM557S type.

Effects of *sbe1* Mutation on the Properties of Endosperm Starch

Starch content in the *sbe1* mutant endosperm was comparable with that of the wild type (Table IV). The apparent amylose content of endosperm starch in EM557S was similar to that in the wild type (Table IV). The λ_{max} values and A_{520}, A_{620}, and A_{680} of iodine-starch complex were not distinguishable between the wild type and *sbe1* mutant (data not shown).

Effects of *sbe1* Mutation on Gelatinization Properties of Starch in Rice Endosperm

The concentration of urea solution for the onset gelatinization was slightly lower in *sbe1* starch than that in rice cv T65 starch (Fig. 5A), although the alkali digestibility was indistinguishable between them (data not shown). When the supernatant from starch solubilized by 4 M urea solution was stained with I_2/KI, the absorption spectra of iodine-starch complex were clearly different among *sbe1*, *ae*, and the wild type (Fig. 5B). The absorbance of iodine-starch complex ranging from 480 nm to 700 nm was highest in *sbe1* mutant and lowest in *ae* mutant. In addition, the λ_{max} value was also highest in *sbe1* mutant and lowest in *ae* mutant. These results suggest that *sbe1* starch granules are soluble in urea solution more easily than wild-type starch granules.

To examine the effects of the mutation in the *Sbe1* gene on the physicochemical properties of starch, thermal properties of starch were analyzed by differential scanning calorimetry (Table VI). The onset gelatinization temperature (T_o) as well as peak (T_p) and conclusion (T_c) temperatures was apparently lower in all the segregated mutant lines as compared with those in the wild-type lines (rice cv T65). The reduc-

T65 (WT) EM557S (*sbe1*) EM529 (*ae*)

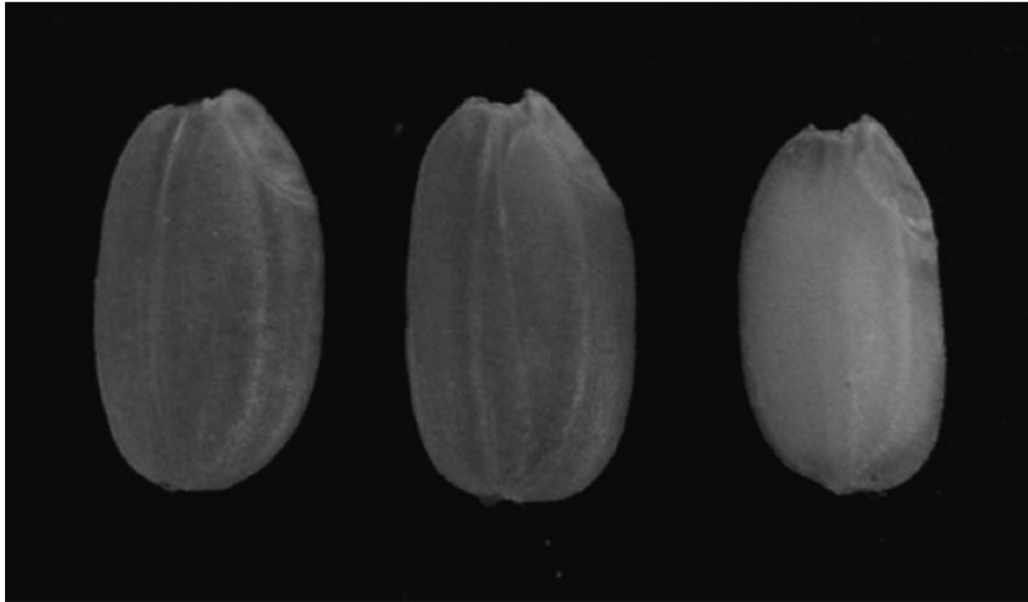

Figure 2. Kernel of rice *sbe1* mutant. Left to right: rice cv T65 (wild type); EM557S (*sbe1* mutant); and EM529 (*ae* mutant).

tion in these parameters due to the *sbe1* mutation was also detected in the mutants having the *wx* background (Table VI). In contrast, *ae* and *ae/wx* starches exhibited higher T_o, T_p, and T_c values. The results strongly suggest that the structural difference in amylopectin induced by *sbe1* mutation affects the gelatinization properties of the starch. The x-ray diffraction pattern of endosperm starch from *sbe1* mutant exhibited the A-type like that of the wild type (data not shown).

DISCUSSION

Recent biochemical and molecular analyses established that green plants have two structurally and functionally distinct types of BE, referred to as BEI and BEII or type B and type A (Burton et al., 1995). This fact tempted us to hypothesize that both types of BE are required for amylopectin biosynthesis.

Previous reports showed that the starches produced in BEIIb-deficient mutants from various plant species such as maize (Stinard et al., 1993), rice (Mizuno et al., 1993), and pea (Bhattacharyya et al., 1990) or in BEIIb-suppressed transgenic potato plants (Safford et al., 1998) are more resistant to gelatinization (Wang et al., 1998; Jane et al., 1999; Nishi et al., 2001). It is considered that resistance to gelatinization is caused mainly by the higher proportion of long chains of amylopectin comprising the starch granules. Recently, Nishi et al. (2001) examined in detail the effect of *ae* mutation on the fine structure of amylopectin in rice endosperm and found that the *ae* amylopectin is specifically depleted in short chains of DP $\leq$ 17 while it has a higher proportion of long chains, consistent with the present study (Fig. 4C).

The present investigation shows that the change in the structure of amylopectin induced by lesion of *BEI* (*Sbe1*) gene was characterized by the specific α-1,4-

Table IV. *Effect of sbe1 mutation on the properties of grain morphology and starches in rice endosperm*

Line	Grain Size				Starch Content		Starch-Iodine Complex		Apparent Amylose Content	
	Length	Width	Thickness	Weight			λ_{max}	BV680[a]	λ_{max}[b]	BV680[c]
		mm			*mg*	%	*nm*		%	
T65	5.4 ± 0.1	3.1 ± 0.1	2.1 ± 0.1	24.3 ± 1.3	18.2 ± 0.5	75.1	561	0.59	16.7	18.9
EM557S	5.3 ± 0.1	3.0 ± 0.1	2.1 ± 0.1	23.4 ± 1.8	17.6 ± 0.3	75.2	562	0.60	16.9	18.9

[a] Blue value (BV) 680, the absorbance at 680 nm of starch-iodine complex. [b] Amylose content determined from the λmax. [c] Amylose content determined from the BV680.

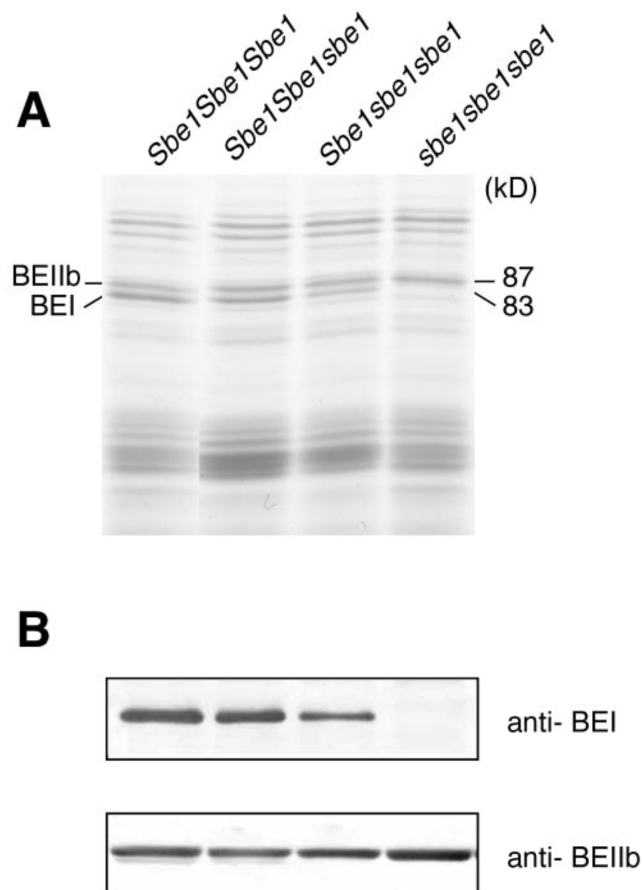

Figure 3. Gene dosage effects of *sbe1* mutation on BE isoforms of rice endosperm. A, SDS-PAGE profile of the crude protein extract of rice endosperm in mature rice kernels. B, Western-blot analysis of BEI and BEIIb in rice endosperm. Protein was extracted from 20 mg of mature rice powder. The immunoblot was developed with antiserum raised against BEI or BEIIb from rice endosperm (Nakamura et al., 1992) at a dilution of 1/1000.

chain length profile in the *sbe1* mutant in that chains of DP 12 to 21 and DP ≥ 37 were depleted, whereas those of DP ≤ 10 and DP 24 to 34 increased (Fig. 4A). The *sbe1* starch had about 6°C lower in the onset gelatinization temperature (T_o) as compared with that of the wild-type starch, whereas *ae* starches showed marked increases in T_o values (Table VI). The fact that the mutant starch was more easily gelatinized is consistent with its better swelling in lower urea concentration (Fig. 5, A and B). The alterations of the amylopectin chain profiles and starch gelatinization properties were considered to be caused by a deficiency of BEI because there were the similar differences in these parameters between amylose-free *sbe1/wx* and *wx* lines examined (Fig. 4B; Table VI). It is stressed that these changes induced by *sbe1* mutation were consistently found in all homozygous mutant lines (Fig. 4; Table VI). The pattern of changes in chain length distribution of *ae* mutation dramatically differed from that of *sbe1* mutation in that in *ae* amylopectin the proportion of short chains of DP ≤ 13 markedly reduced, whereas that of long chains of DP ≥ 15 increased (Fig. 4C). It was found that the level of BEIIb was found to be only slightly higher (about 113%) in *sbe1* mutant as compared with that of wild type (Fig. 3). Therefore it is likely that the alteration of the structure of amylopectin and the starch gelatinization properties found in *sbe1* mutant of rice was caused by a deficiency in BEI activity, although one cannot exclude the possibility that these phenotypic changes were partly due to the elevated level of BEIIb activity.

Table V. *Activities of SS and AGPase in developing rice endosperm of BEI-deficient mutant EM557S and its parent rice cv T65*

All enzymatic activities were measured using linear standard condition, expressed as nanomoles per minute per endosperm. The values are means ± SD of at least three replicate measurements from a representative experiment.

Enzyme	T65	EM557S
SS	14.0 ± 0.6	16.0 ± 0.4
AGPase	124 ± 1	130 ± 2

Blauth et al. (2002) reported that a BEI-lacking mutant of maize synthesizes the same chain profile for amylopectin as the wild type. The reason for the phenotypic differences between the two maize and rice mutants is unknown at present. It is possible that there are some differences in kinetic parameters and/or specificities among BE isoforms from maize and rice and/or that the multiple BEI-type isoforms that can functionally complement each other are present in maize endosperm, although other possibilities cannot be ruled out.

The differences in the amylopectin structure between *sbe1* and *ae* mutants were markedly reflected on the differences in physicochemical properties of the starches between the two mutants. First, the *ae* starch was hard to gelatinize, whereas *sbe1* starch was easily gelatinized (Table VI; Fig. 5). Second, x-ray diffraction pattern analysis showed that the *ae* starch was converted to the B-type starch from the A-type starch present in wild type, whereas the *sbe1* starch maintained the A-type starch (data not shown).

The *sbe1* mutant exhibited the following notable phenotypes. Although the *sbe1* mutant had an altered amylopectin structure, the extent of the change was not so drastic as observed in the *ae* mutant (Fig. 4). Although long (B) chains with DP ≥ 37 were significantly decreased by the *sbe1* mutation, a sufficient amount of long chains still remained. These results suggest that the formation of long chains by BEI can be complemented, at least partly, by BEIIb and/or BEIIa, whereas the formation of short chains, A chains, is specifically catalyzed by BEIIb (Nakamura, 2002; Nakamura et al., 2003).

Nakamura et al. (1994) located the BEI gene on chromosome 6 in the rice genome by gene mapping analysis. The *sbe1* mutant used in this study was

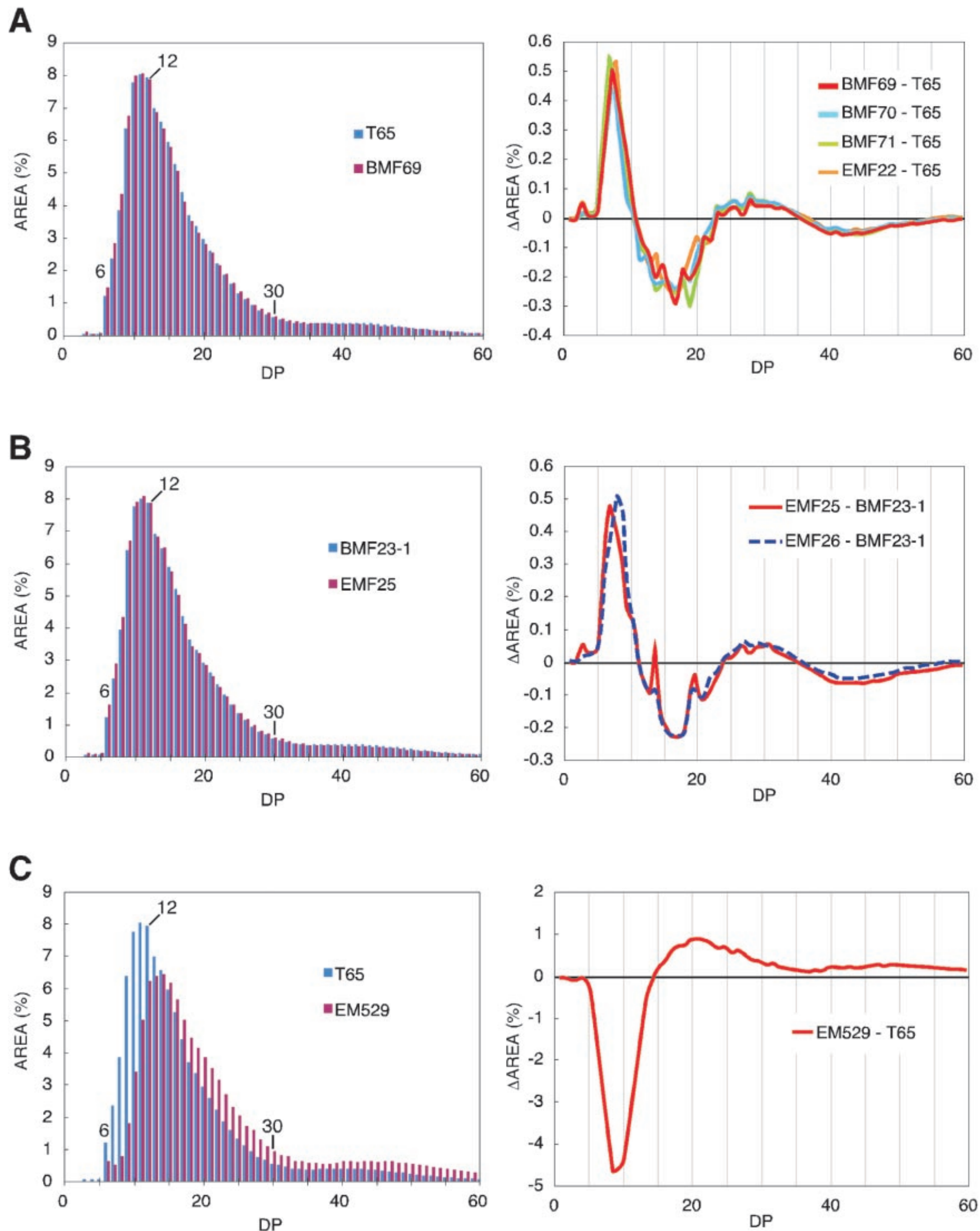

Figure 4. Effect of *sbe1* mutation on chain length distribution of amylopectin in endosperm from BEI-deficient mutant of rice as determined by APTS-capillary electrophoresis. Chain length distributions were determined by analyzing APTS-labeled debranched starches on the molar basis, and the peak area of a fraction of linear chain with a specific chain length was calculated as a percentage of total peak area up to DP of 80, although the figures present the results up to DP 60 only. A, The distribution of α-1,4-glucan chains in amylopectin from wild-type rice cv T65 and the four homozygous *sbe1* mutant lines, BMF69, BMF70, BMF71 (EM557S), and EMF22. The data for rice cv T65 were the averages of those for three different homozygous rice cv T65 lines, although no substantial differences among them were detected. B, The chain distribution of amylopectin from two *sbe1*/*wx* lines (EMF25 and EMF26) and two *wx* mutant lines (BMF23-1 and BMF23-2). C, The chain distribution of amylopectin from rice cv T65 and *ae* mutant EM529. The data for rice cv T65 were the averages of those for three different homozygous rice cv T65 lines. Figures on the right show differences between the respective mutants and rice cv T65 (A and C) or the *wx* mutant line BMF23-1 (B). The data shown are representatives from three experiments that gave similar results. For isolation of homozygous mutant and wild-type lines, see "Materials and Methods" in detail.

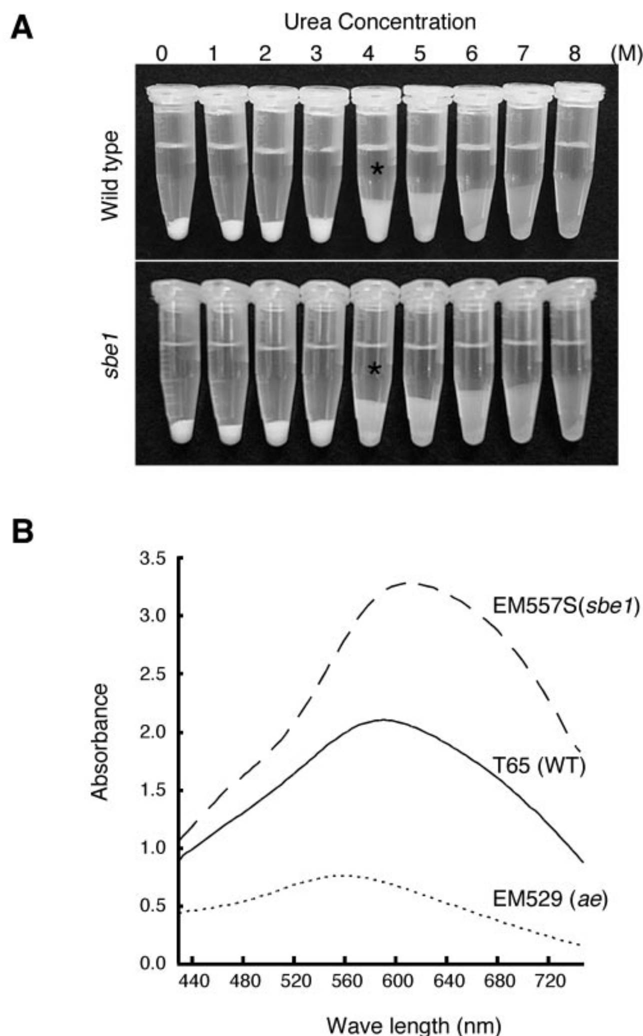

Figure 5. Effect of *sbe1* mutation on gelatinization properties of starch from endosperm of mature seed. A, Gelatinization of starch from the *sbe1* mutant and wild type in various concentrations of urea solution. Ten milligrams of rice powder in an Eppendorf tube was mixed with 1 mL of urea solution and shaken for 24 h at 25°C. After centrifugation, samples were allowed to stand for 1 h. B, Absorbance spectra of resolved starches in the I_2/KI solution. These starches were obtained in the supernatant after treatment with 4 M urea solution as shown by asterisks in A.

caused by lesion of a single gene (Table I), and the *sbe1* gene was determined to be present on chromosome 6 based on the trisomic analysis (Table II). It was also found that the *sbe1* gene was linked to the *Ur* marker gene that is known to be located in the long arm of chromosome 6, close to the position of the *BEI* gene (data not shown). On top of these data, our RFLP analysis using an EST marker (accession no. EC0727) encoding BEI indicates that *sbe1* was caused by mutation of the BEI gene on chromosome 6 of rice (Table III).

We reported previously that the *flo2* mutation of rice reduces the expression of BEI to less than one-tenth of that in the wild type (Kawasaki et al., 1996). This mutation coregulates the expression of other enzymes involved in the starch biosynthesis in endosperm to less than 70% of the wild-type levels except for AGPase. The *flo2* mutation exhibits an opaque brown phenotype and reduces the amount of starch (Satoh and Omura, 1981). The loss of BEI in the *sbe1* mutation, however, did not affect the accumulation of starch and the morphological properties not only of the grain but the plant as a whole (Fig. 2; Table IV). These results show that a low level of BEI per se does not cause the *flo2* phenotype in endosperm and that the alteration of the amylopectin structure can be induced only when BEI activity is almost or completely lacking (Fig. 4A), notwithstanding the fact that the BEI activity accounts for about 60% of the total BE activities in endosperm (Yamanouchi and Nakamura, 1992).

There have been several investigations to characterize BE isoforms and compare BE isoforms with each other and with the glycogen-branching enzyme (GBE). For example, maize BEI preferentially produces longer chains, whereas BEII generates shorter chains; and the minimum chain length required for BEI is presumably DP 16, whereas that for BEII is DP 11 to 12, the same value for GBE (Takeda et al., 1993; Guan et al., 1997). Maize endosperm BEI and BEII can also produce chains with a wide range of chain lengths over DP 33 as compared with GBE, which effectively forms a narrower range of short chains of DP 6 to 20 when these enzymes are incubated with amylose of DP 405 (Takeda et al., 1993; Guan et al., 1997). These observations strongly suggest that BEI and BEII play distinct roles in formation of long and short chains of amylopectin, respectively.

The structure of amylopectin in higher plants is characterized by the fact that a unit structure with a constant size throughout the plant kingdom called cluster is tandem linked (Jenkins et al., 1993; Thompson, 2000), and the distinct structure may be referred to as "tandem-cluster structure." The individual cluster has an amorphous region and a crystalline region, and branches are distributed in the both regions (Hizukuri, 1996; Bertoft and Koch, 2000). On the basis of these observations, it is reasonable to assume that BE should produce three different types of branches that contribute to the formation of the amylopectin structure: those formed in the amorphous and the crystalline regions of the cluster, and those that link the clusters.

Bertoft and Koch (2000) proposed that the sizes for A, B_1, and B_{2-3} chains of amylopectin in rice endosperm are in the range of DP $\leq$ 17 to 18, DP 18 to 28, and DP $\geq$ 28, respectively. If this is the case, differences between chain length distribution of amylopectins from *ae* and *sbe1* mutants of rice supports the view that BEIIb and BEI play important roles in the formation of A chains, and B_1 chains and cluster-connecting B chains of amylopectin, respectively (Fig. 4).

Table VI. *Thermal properties of starch in endosperm of BEI-deficient mutant of rice as measured by differential scanning calorimetry*

Four homozygous *sbe1* mutant lines, BMF69, BMF70, BMF71 (EM557S), and EMF22; two homozygous *wx* mutant lines, BMF23-1 and BMF23-2; two homozygous *sbe1/wx* mutant lines, EMF25 and EMF26; a homozygous *ae* mutant line, EM529; and a homozygous *ae/wx* mutant line, AMF24 were used for analyses as well as the wild-type rice cv T65. The values are averages of three replications.

Genotype	Line	T_o[a]	T_p[b]	T_c[c]	DH[d]
			°C		$J\ g^{-1}$
Sbe1Sbe1/WxWx	T65	51.5 ± 0.5	60.3 ± 0.2	68.7 ± 1.3	10.3 ± 1.4
sbe1sbe1/WxWx	BMF69	45.9 ± 0.6	55.2 ± 0.2	64.7 ± 1.2	10.0 ± 0.4
sbe1sbe1/WxWx	BMF70	45.5 ± 0.3	55.0 ± 0.3	63.9 ± 0.4	9.3 ± 0.3
sbe1sbe1/WxWx	BMF71	45.1 ± 0.2	55.0 ± 0.3	63.9 ± 0.9	9.2 ± 0.4
sbe1sbe1/WxWx	EMF22	45.8 ± 0.3	55.4 ± 0.3	64.6 ± 0.3	8.3 ± 0.5
Sbe1Sbe1/wxwx	BMF23-1	51.9 ± 0.8	61.3 ± 0.5	68.5 ± 0.3	13.1 ± 0.5
Sbe1Sbe1/wxwx	BMF23-2	53.1 ± 0.4	62.9 ± 0.4	71.0 ± 0.6	15.0 ± 0.4
sbe1sbe1/wxwx	EMF25	43.4 ± 0.5	54.8 ± 0.3	63.7 ± 0.2	10.0 ± 0.4
sbe1sbe1/wxwx	EMF26	46.4 ± 0.4	55.7 ± 0.2	65.4 ± 0.8	11.3 ± 0.6
aeae/WxWx	EM529	64.4 ± 1.1	76.0 ± 0.2	83.5 ± 0.4	8.9 ± 0.6
aeae/wxwx	AMF24	70.2 ± 0.3	77.6 ± 0.0	83.9 ± 0.3	11.7 ± 0.4

[a] Onset temperature. [b] Peak temperature. [c] Conclusion temperature. [d] Gelatinization enthalpy of starch.

Finally, it should be pointed out that the manipulation of BEI gene could be useful for production of novel starches with different functional properties in rice endosperm (Fig. 5; Table VI), although the *sbe1* mutation did not bring about apparent phenotypic changes in the morphology and the starch content of the seed (Fig. 2; Table IV), in contrast with other starch mutants, such as *ae*, *sug1*, *wx*, *shr*, and *flo*.

MATERIALS AND METHODS

Plant Materials

One thousand and forty one endosperm mutant lines used in this experiment were stocked at the Plant Genetics Laboratory of Institute of Genetic Resources, Faculty of Agriculture, Kyushu University (Japan). These mutant lines were produced by the treatment of fertilized egg cells of *japonica* rice (*Oryza sativa* cvs Kinmaze and T65) with MNU as reported by Satoh and Omura (1979). Rice plants were grown at an experimental field of Kyushu University Farm under natural condition.

Seeds used for physiological and biochemical analyses were set on plants of the BC_2F_3 generation, which were produced as follows. A mutant line having *sbe1* and *flo3* mutations, EM557, which was induced by the MNU treatment with fertilized egg cells of a *japonica* rice cv T65 was crossed to rice cv T65. F_1 plants were self-pollinated and then F_2 seeds showing normal phenotype in grain appearance were grown and self-pollinated. F_2 plants to be homozygous for *sbe1* were isolated by SDS-PAGE and native-PAGE/activity staining analyses of the developing seeds, and they were crossed again to rice cv T65. Homozygous *sbe1* plants were selected in F_2 population from the cross via SDS-PAGE and native-PAGE/activity staining analyses of the developing seeds, and self-pollinated. Homozygous *sbe1* plants of three BC_2F_3 lines, BMF69, BMF70, and BMF71, derived from the two independent crossings were used for endosperm analysis. In addition, homozygous *sbe1* plants of the BC_1F_5 progeny line, EMF22, were used for endosperm analysis. In this study, the BMF71 was referred to as EM557S and was used as the standard *sbe1* mutant line.

F_2 plants determined to be recessive homozygous *sbe1* were crossed to a *wx* mutant line, EM583, which was also induced by the MNU treatment of rice cv T65 and was deficient in GBSS. More than 50 F_2 plants to be homozygous for *wx* derived from the cross were self-pollinated, and 14 F_2 plants were determined to be homozygous for BEI deficiency via SDS-PAGE and native-PAGE/activity-staining analyses of the developing seeds. Two independent progeny lines, EMF25 and EMF26, to be homozygous for *sbe1/wx* (amylose-free BEI-deficient plant line) were self-pollinated through two generations for endosperm starch analysis. In addition to EMF25 and EMF26, two homozygous *wx* lines, BMF23-1 and BMF23-2, were used for analyses for amylopectin chain length distribution and starch gelatinization analyses. An *ae* mutant line being defect in BEIIb, EM529, which was also induced by the MNU treatment of rice cv T65 was used for comparing the effects of BE isoforms on the structure of amylopectin and physicochemical properties of the starch. AMF24, an amylose-free *ae/wx* mutant line, was derived from a cross between EM529 (*ae*) and EM583 (*wx*).

Preparation of Enzyme Extract

For assay of enzyme activities, developing endosperm at the late milky stage was removed from embryo and pericarp and was homogenized with 10 mL of an extraction buffer containing 50 mM HEPES-NaOH (pH 7.4), 4 mM $MgCl_2$, 50 mM 2-mercapthoethanol, and 12.5% (v/v) glycerol. The homogenate was centrifuged at 10,000*g* for 10 min at 4°C. The supernatant referred to as the soluble enzyme extract was used for enzyme assay and zymogram analysis.

For screening of BEI-deficient mutants, the total proteins were extracted from one mature brown rice seed for each mutant line. The seed was crushed by pliers, and the tissue was placed into a micro test tube (1.5 mL) and homogenized with 500 μL of a buffer containing 8 M urea, 4% (w/v) SDS, 5% (v/v) 2-mercapthoethanol, and 0.125 M Tris-HCl (pH 6.8). The homogenate was shaken at 100 rpm for at least 4 h, and centrifuged at 15,000*g* for 5 min at 15°C. The supernatant referred to as the protein extract was used for SDS-PAGE analysis.

SDS-PAGE and Immunoblotting and Northern Blotting

SDS-PAGE of the crude protein extract and western blotting and northern blotting were performed as described previously (Nishi et al., 2001).

Genetic Analysis of BEI-Deficient Mutant EM557

Southern blotting was performed according to the protocol of ECL kit (Amersham Biosciences UK, Little Chalfont, Buckinghamshire, UK) using a

cDNA clone (EC0727) as a probe. Trisomic analysis was performed by crossing nine types of trisomic plants with EM557S.

The endosperm gene dosage series was generated by self or reciprocal crosses within or between homozygous *Sbe1 Sbe1* (rice cv T65) and *sbe1 sbe1* (EM557S) parents. Endosperms containing 3, 2, 1, and 0 copies of *Sbe1* gene were those from the self-pollinated rice cv T65, rice cv T65 (female) × EM557S (male), EM557S (female) × rice cv T65 (male), and the self-pollinated *sbe1* mutant strain EM557S, respectively.

Zymogram Analysis of BEs and Starch-Debranching Enzymes

Zymogram analysis was performed as described previously (Nishi et al., 2001).

Determination of Amylose Content by Iodine Calorimetric Analysis

Rice starch was prepared from mature seeds of the wild type and the *sbe1* mutants by the diluted-alkali method (Yamamoto et al., 1973) and gelatinized by treatment with 1 N NaOH. Amylose content was measured by the colorimetric method (Juliano, 1971), using an iodine-potassium iodide solution. Twenty grains of average-sized brown rice were polished to remove the embryo and pericarp by a test mill Pearlest (Kett, Tokyo). After measuring the weight, three polished rice grains of each strain were put individually into a 20-mL test tube containing 2 mL of 1 N NaOH and incubated at room temperature about 25°C for 24 h. The solution containing the alkaline-digested starch was neutralized with 4 mL of 1 N CH_3COOH, filled up to 10 mL by adding 4 mL of distilled water and then homogenized by an Ultrasonic Disrupter (Tomy, Tokyo). A 0.8-mL aliquot of the crude starch solution was stained by 0.2 mL of an iodine solution containing 0.2% (w/v) iodine and 2% (w/v) potassium iodine and diluted with 4 mL of distilled water. The characteristics of the iodine-starch complex were measured colorimetrically using a spectrophotometer model 7000 (Beckman Coulter, Fullerton, CA). Apparent amylose content was estimated by the method of Juliano (1971).

Assay of Enzymes

The activities of AGPase and SS were assayed as described previously (Nishi et al., 2001).

Chain Length Profile of Amylopectin

Analysis of the chain length distribution of amylopectin isoamylorysates was performed with a modification of the method of O'Shea et al. (1998), as described previously (Nakamura et al., 2002).

Measurement of Gelatinization Properties

The gelatinization and swelling modes of endosperm starch in variable concentrations of urea were measured as described previously (Nishi et al., 2001). The solubility of starch granules in urea solution was expressed in terms of the absorbance of the iodine-starch complex of supernatant from 4 M urea solution. The thermal gelatinization properties of starch were analyzed by differential scanning calorimeter.

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