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Granule-Bound Starch Synthase I is Responsible for Biosynthesis of Extra-Long Unit Chains of Amylopectin in Rice

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A rice *Wx* gene encoding a granule-bound starch synthase I (GBSSI) was introduced into the null-mutant *waxy* (*wx*) rice, and its effect on endosperm starches was examined. The apparent amylose content was increased from undetectable amounts for the non-transgenic *wx* cultivars to 21.6–22.2% of starch weight for the transgenic lines. The increase was in part due to a significant amount of extra-long unit chains (ELCs) of amylopectin (7.5–8.4% of amylopectin weight), that were absent in the non-transgenic *wx* cultivars. Thus, actual amylose content was calculated to be 14.9–16.0% for the transgenic lines. Only slight differences were found in chain-length distribution for the chains other than ELCs, indicating that the major effect of the *Wx* transgene on amylopectin structure was ELC formation. ELCs isolated from debranched amylopectin exhibited structures distinct from amylose. Structures of amylose from the transgenic lines were slightly different from those of cv. Labelle (Wx^a) in terms of a higher degree of branching and size distribution. The amylose and ELC content of starches of the transgenic lines resulted in the elevation of pasting temperature, a 50% decrease in peak viscosity, a large decrease in breakdown and an increase in setback. As yet undetermined factors other than the GBSSI activity are thought to be involved in the control of formation and/or the amount of ELCs. Structural analysis of the *Wx* gene suggested that the presence of a tyrosine residue at position 224 of GBSSI correlates with the formation of large amounts of ELCs in cultivars carrying Wx^a.

Keywords: Amylopectin — Extra-long chains — Granule-bound starch synthase — Rice — Starch — *Wx*.

Abbreviations: CL, chain length; DP, degree of polymerization; ELC, extra-long (unit) chain; GBSSI, granule-bound starch synthase I; HPSEC, high-performance size-exclusion chromatography; SNP, single nucleotide polymorphism.

Introduction

Starch, a major component of cereal grains, is comprised mainly of two types of α-glucans, amylose and amylopectin. The former is made of linear or slightly branched molecules and the latter is composed of highly branched molecules (Tester et al. 2004, Hizukuri et al. 2006). It is well known that the amylose content of starch granules is related to some of the properties of starch and cooked grains. Thus manipulation of the amylose content through various means has been attempted in order to generate starches that exhibit unique properties. Depending on the experimental method used for determination of amylose in starch samples, the amylose content is generally expressed as either apparent or actual content (Takeda et al. 1987). Apparent amylose content can be obtained by methods such as iodine-binding capacity measurement or gel permeation chromatography of debranched starch. Some amylopectins have been shown to have a fraction of unit chains that have a chain length long enough to behave similarly to amylose in these measurements of amylose content. Thus, actual content is derived from apparent content by subtracting the contribution of the long chains of amylopectin (Takeda et al. 1987).

An enzyme responsible for amylose synthesis in vivo is granule-bound starch synthase (GBSS), which is usually found as a starch synthase isozyme bound to or embedded in starch granules. In rice, GBSSI is encoded by the *Wx* gene (Okagaki 1992, Hirose and Terao 2004) which has two functional alleles, Wx^a and Wx^b (Sano 1984). Wx^b carries a substitution mutation at the 5′ splice site of the first intron that results in small amounts of *Wx* mRNA (Isshiki et al. 1998, Cai et al. 1998, Hirano et al. 1998, Larkin and Park 1999) and GBSSI in developing endosperm, and a slightly lower apparent amylose content of starch than that of Wx^a (Sano et al. 1984). Wx^a is mainly distributed in Indica cultivars of *Oryza sativa* L., *O. glaberrima* and their wild progenitors, while Wx^b is distributed in Japonica cultivars of *O. sativa* L. (Hirano et al. 1998). Starches from the two major

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groups of rice, Indica and Japonica, have been examined, and it was observed that some Indica cultivars show much higher apparent amylose content than Japonica cultivars, but the actual content is comparable between the two groups (Takeda et al. 1987). The difference was due to the presence of unusually long chains [extra-long chains (ELCs) or also designated as super-long chains (SLCs)] in amylopectins of the Indica rice starches (Takeda et al. 1987). This finding and other published data (Hizukuri et al. 1989, Inouchi et al. 2005) indicated that despite carrying a functional *Wx* allele, many of the Japonica cultivars lack ELCs of amylopectin.

There is accumulating evidence that GBSS is also involved in amylopectin synthesis, especially in forming the ELC fraction. For example, GBSS has been reported to act on a specific subset of amylopectin unit chains to form ELCs (Baba et al. 1987, Denyer et al. 1996). The involvement of GBSS in amylopectin synthesis has also been suggested by the fact that the loss of GBSS expression leads to loss of the ELC fraction of amylopectin (Hizukuri et al. 1989). Reduction or complete loss of the ELC fraction in amylopectin by reduction of GBSSI activity was also observed, for example, in wheat (Yoo and Jane 2002), sweet potato (Kitahara et al. 2007) and *Chlamydomonas* (Maddelein et al. 1994).

Recently, Itoh et al. (2003) reported that introduction of *Wx*::*Wx*^{a} cDNA (pWxR) into the null-mutant *wx* rice leads to production of starches with high apparent amylose content. In this study, structures of the starches from the transgenic rice were examined in detail after fractionation into amylose and amylopectin, and compared with the non-transgenic *wx* cultivars and a *Wx*^{a}-containing cultivar to clarify the role of GBSSI in starch biosynthesis in rice endosperm.

Results

Amylose contents

Introduction of a *Wx*::*Wx*^{a} cDNA (*WxR*) fusion gene into the null-mutant *wx* rice resulted in transgenic lines (*WxR*/*wx*) I26, M27 and M214, that produced endosperm starches with high apparent amylose content (Itoh et al. 2003). The *WxR* transgene carries the first intron of *Wx*^{a}, and the mRNA and protein of the *WxR* are identical to those of *Wx*^{a}. Starches from cv. Labelle (*Wx*^{a}), Iwaimochi (*wx*), Musashimochi (*wx*) and the transgenic lines were debranched with isoamylase, and the hydrolyzates were analyzed by high-performance size-exclusion chromatography (HPSEC) after fluorescent labeling (Fig. 1). Generally, the longest chain fraction in debranched starch (in this case, eluted at a retention time of ~60–70 min) is regarded as amylose. However, long unit chains of amylopectin can be present in the same elution fraction. Thus, the percentage of the peak area as indicated in the figure was considered as apparent amylose content (Table 1). Introduction of the *WxR* gene into the null-mutant *wx* caused a drastic increase in apparent amylose content of endosperm starches, from an undetectable amount in the non-transgenic *wx* to ~22% in the transgenic lines.

To clarify the possible contribution of long unit chains of amylopectin to the apparent amylose content, fractionated amylopectin was similarly analyzed (Fig. 2). Amylopectins from the three transgenic lines had unit chains that eluted at the same position as amylose, and these extremely long chains were absent in the non-transgenic *wx* starches. Thus, apparent amylose of the transgenic lines includes these long chains of amylopectin in addition to amylose.

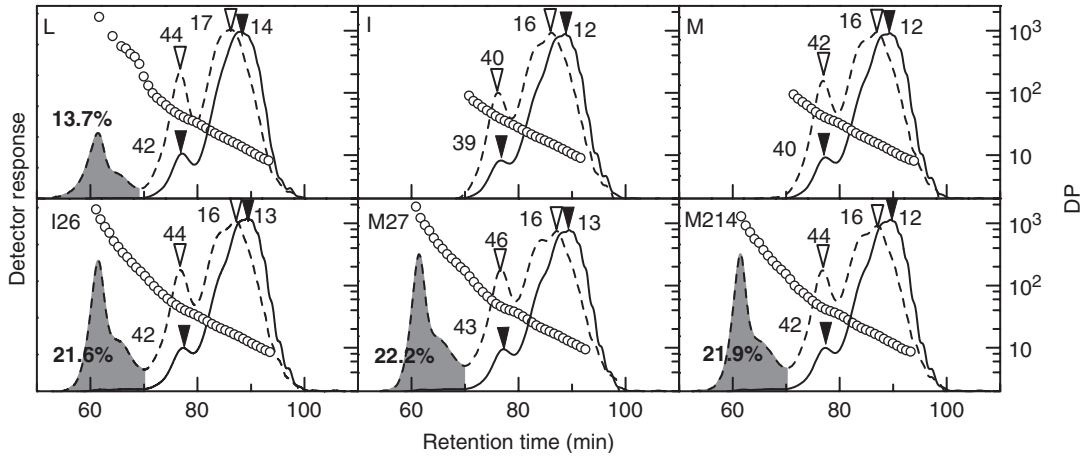

Fig. 1 Chain-length distributions of the rice starches. Starch was debranched with isoamylase and the hydrolyzate was subjected to fluorescent labeling of the reducing terminus followed by high-performance size-exclusion chromatography (HPSEC) with a HPLC system equipped with fluorescence and refractive index detectors. Solid line, fluorescence (a molar basis); dashed line, refractive index (a weight basis); open circle, degree of polymerization (DP). Arrowheads indicate the DP at given elution positions. The peak corresponding to apparent amylose is shown in gray.

Structure of amylopectin

The extremely long chains that were regarded as a part of apparent amylose in the chain-length distributions profiles of amylopectins (Fig. 2) were designated as ELCs, and their contents in amylopectin were compared (Table 2). On a weight basis, a significant amount of ELCs was found in the transgenic lines but not in the two *wx* cultivars, Iwaimochi and Musashimochi, and only a slight amount in cv. Labelle. Debranched amylopectin was fractionated by 1-butanol precipitation to prepare and characterize the ELC fraction. The number-average degree of polymerization (DPn) and DP distribution of ELCs determined by the labeling/HPSEC were very similar among the ELCs of the transgenic lines examined. The DPn was 380, 350 and 390 for line I26, M27 and M214, respectively. Peak DP in molar- and weight-based distributions, respectively, were 250 and 320 for line I26, 240 and 310 for line M27, and 220 and 280 for line M214. The result for line I26 is shown as an example in Fig. 3. These structural characteristics of ELC were clearly different from those of amylose fractionated from the same starch specimen. The DPn of ELCs was much smaller than that of amylose, and the DP distribution of ELCs was much narrower and shifted to a lower DP range compared with amylose. The structures of the ELCs were very similar to those described in a previous study on ELCs from rice, wheat, maize, buckwheat and sweet potato amylopectins (Hanashiro et al. 2005).

Table 1 Amylose contents of starches of different rice cultivars (L, I and M) and transgenic rice lines (I26, M27 and M214)

Source	Amylose content (% of total starch by weight)	
	Apparent	Actual[a]
Labelle (L)	13.7	13.1
Iwaimochi (I)	nd	nd
I26	21.6	15.0
Musashimochi (M)	nd	nd
M27	22.2	16.0
M214	21.9	14.9

[a]The actual amylose content was obtained by overlaying chromatograms of debranched starch and amylopectin, and subtraction of the peak area of ELCs of amylopectin from the corresponding peak of debranched starch. The subtraction was done after normalization of the peak area of unit chains shorter than ELCs with the assumption that these short unit chains (A, B_1, and B_2+B_3 fractions in Table 2) arose only from amylopectin.
nd, not detected.

In addition to the changes in the formation of the ELC fraction, the composition of unit chains was also modified by the transgene expression (Fig. 2, Table 2). As shown in the upper right panel in Fig. 2, unit chain fractions are designated according to their length as A, B_n and ELC fractions. A chains carry no side chains and link to other amylopectin chains at the reducing terminus *via* α-1, 6-linkage, while B chains carry side chains and link to other amylopectin chains in the same manner as A chains (Peat et al. 1952, Peat et al. 1956). The subscript in B_n indicates the number of clusters in which the B chain is involved (Hizukuri 1986). On a weight basis, the percentage of A and B_1 fractions was decreased in the transgenic plants, while the B_2+B_3 fraction was essentially unchanged in spite of the presence of a newly formed ELC fraction. On a molar basis, the B_2+B_3 fraction was slightly increased and a concomitant decrease of either the A or B_1 fractions was found. As a result, the molar ratio of $(A+B_1)/(B_2+B_3)$ was slightly decreased in the transgenic lines.

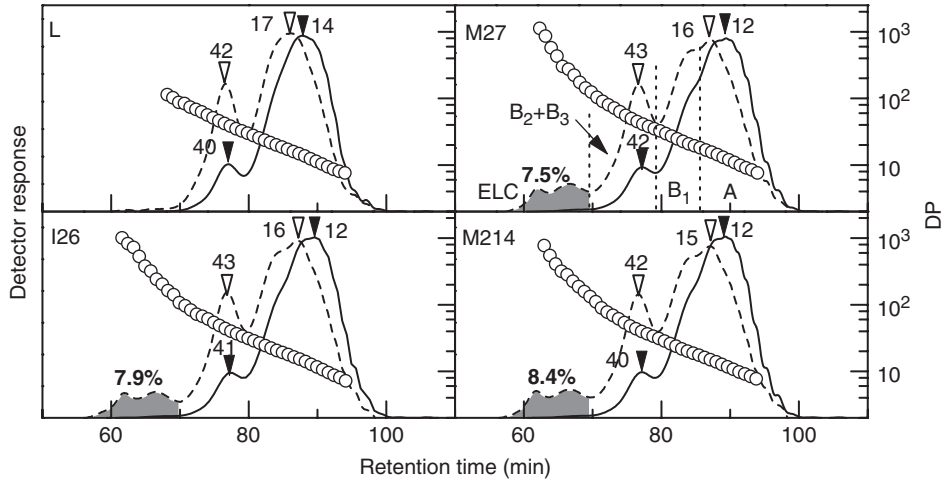

Fig. 2 Chain-length distributions of the rice amylopectins. Amylopectin was debranched with isoamylase and analyzed with HPSEC as described in the legend for Fig. 1. The elution profile was divided into A, B_1, B_2+B_3 and ELC fractions according to chain length. Symbols are as shown in Fig. 1. The peak corresponding to the ELC fraction was shown in gray.

Table 2 Distribution of different amylopectin chain lengths (A, B_1, B_2, B_3 and ELCs) in different rice cultivars (L, I and M) and transgenic rice lines (I26, M27 and M214)

Source	Amount by weight (%)				Amount by mol (%)				Molar ratio, $(A+B_1)/(B_2+B_3)$
	A	B_1	B_2+B_3	ELC	A	B_1	B_2+B_3	ELC	
L	73.3[a]		26.1	0.6	89.0[a]		11.0	nd	8.1
I	47.4	31.0	21.6	nd	68.8	22.8	8.4	nd	10.9
I26	41.2	28.2	22.7	7.9	67.0	23.2	9.3	0.5	9.7
M	45.6	30.9	23.5	nd	67.6	23.5	8.9	nd	10.2
M27	42.0	26.8	23.7	7.5	67.7	22.2	9.6	0.5	9.4
M214	41.1	27.4	23.1	8.4	66.7	23.4	9.5	0.4	9.5

[a]The sum of the A and B_1 fractions is given due to difficulty in dividing them into the two fractions. nd, not detected. L, cv. Labelle; I, cv. Iwaimochi; M, cv. Musashimochi.

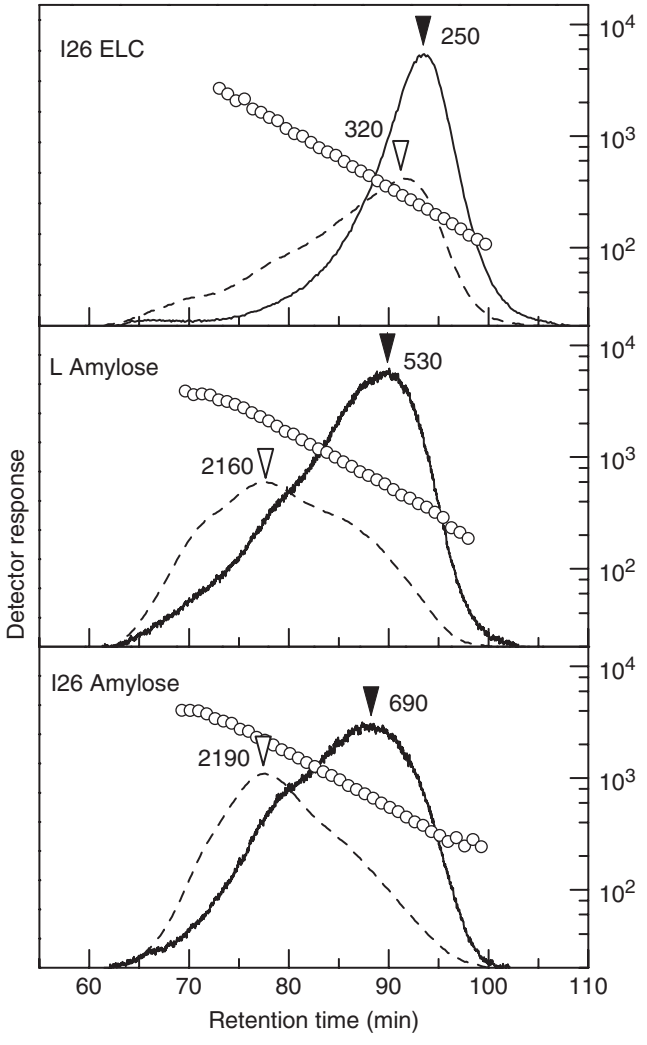

Fig. 3 DP distributions of ELCs of amylopectin of line I26 and amyloses of cv. Labelle (L) and I26. ELCs isolated from debranched amylopectin and amylose were analyzed with HPSEC after fluorescent labeling. Symbols are as shown in Fig. 1.

Structure of amylose

The structures of amyloses obtained from starches of cv. Labelle and the transgenic lines are summarized in Table 3. DPn, which is a measure of the average size of molecules, was essentially similar among the amyloses examined and comparable with those reported previously for rice (Takeda et al. 1986). Since amylose is a mixture of linear and slightly branched molecules, branching characteristics, such as how many chains on average comprise one molecule (expressed as the number of chains, NC), how many glucosyl residues on average comprise one unit chain (expressed as number-average chain length, CLn) and the ratio of linear and branched molecules by mole (expressed as molar fraction of branched molecules, MF_B), of amyloses were also examined. A large difference was observed in the CLn and NC between cv. Labelle and the transgenic lines. The difference was due to the increase in branched molecules on a molar basis, as indicated by the almost doubled MF_B, and increased number of chains of branched molecules (NC_B). DP distributions of Labelle and I26 amyloses are shown in Fig. 3. The distributions of the transgenic starches were very similar to each other. Peak DPs in molar- and weight-based distributions were 690 and 2,310 for M27 amylose, and 690 and 2,270 for M214 amylose. Consistent with the DPn values, the DP distribution was shifted toward the higher DP range (leftward), and the molar proportion of a slight shoulder around retention time 75–80 min was slightly higher for the transgenic lines.

Pasting properties of starch granules

Viscograms (Fig. 4) and the characteristic values (Table 4) differed significantly between the non-transgenic *wx* cultivars and the transgenic lines. Expression of GBSSI caused elevation of pasting temperature and of the temperature at maximum viscosity, a decrease in maximum viscosity, reduction of breakdown and an increase in setback. Differences in pasting temperature between the

Table 3 Properties of amyloses in cultivar Labelle (L) and transgenic lines (I26, M27 and M214)

Source	DPn		CLn	NC	MF_B	NC_B[a]
	Colorimetric	Labeling				
L	920	1190	380	2.4	0.22	7.4
I26	1040	1240	210	5.0	0.41	10.8
M27	940	1220	210	4.5	0.43	9.1
M214	910	1220	200	4.6	0.44	9.2

DPn, number-average degree of polymerization; CLn, number-average chain length; NC, average number of chains; MF_B, molar fraction of branched molecules; NC_B, average number of chains of branched molecules.
[a]Calculated using the following equation: $NC_B = [NC - (1 - MF_B)]/MF_B$.

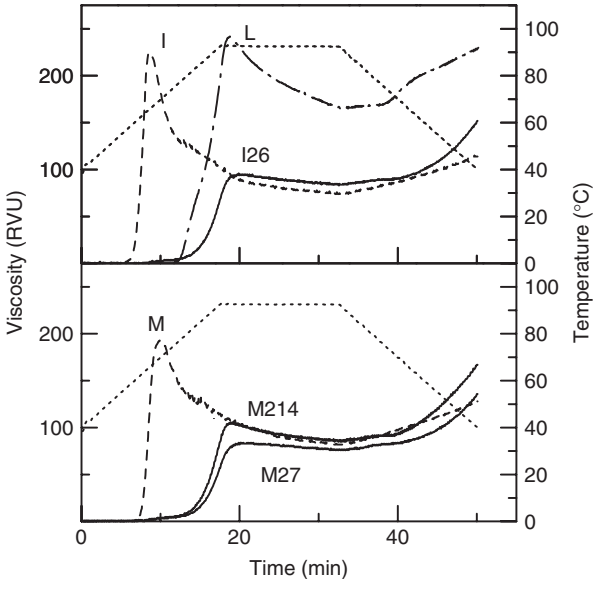

Fig. 4 Viscograms of the rice starches. Pasting properties of starch (9% w/w) were analyzed with a Rapid Visco Analyzer. Top panel, cv. Labelle (L), and cv. Iwaimochi (I) and corresponding transgenic line I26; bottom panel, cv. Musashimochi (M) and corresponding transgenic lines M27 and M214. Solid line, transgenic line starch; dashed line, non-transgenic *wx* starch; dotted and dashed line, cv. Labelle; dotted line, temperature.

non-transgenic *wx* cultivars and transgenic lines are approximately 5–10°C. Viscograms of the transgenic starches showed a significant delay of the rapid increase phase after the initial rise in viscosity while in Labelle and the non-transgenic *wx* cultivars the viscosity rapidly reached the maximum value once it started to rise. From the slope of the viscosity curve near its maximum, the pasting temperature of the transgenic starches could be estimated at 85–87°C. Maximum viscosity of the transgenic starches was reduced almost by half compared with the non-transgenic *wx* and Labelle cultivars. Breakdown, which is a measure of the degree of fragmentation of swollen granules by shear force, was greatly reduced in the transgenic starches. Setback, which indicates the tendency of the starch paste to retrograde, increased in the transgenic lines when compared with the corresponding non-transgenic *wx* cultivars, and the values for the I26 and M214 lines were also higher than that of cv. Labelle.

Discussion

Effect of the Wx^a transgene on amylose content of starch

In a previous study (Itoh et al. 2003), we showed that starches from the transgenic lines carrying the *WxR* gene had a high apparent amylose content by iodine staining of starch granules. The detailed structural characterization of amylose and amylopectin fractionated from starches described in this study clearly demonstrates that their high content was not attributable simply to an increase in amylose, and that amylopectin structure was also modified by GBSSI expression, revealing unambiguously that GBSSI is directly involved in synthesis of amylopectin. The major involvement of GBSSI was in the addition of ELC onto amylopectin molecules. This ELC fraction of amylopectin unit chains contributed to the observed increase in apparent amylose content. The actual amylose content was only slightly higher in the transgenic lines compared with cv. Labelle (Table 1). As Umemoto et al. (2002) reported, we observed in a previous study that the apparent amylose content was not proportional to the relative amount of Wx protein in isolated starch granules (Itoh et al. 2003). Similarly, the actual amylose content and ELC content in amylopectin (Table 2) was comparable among the transgenic lines regardless of the Wx protein levels. Assuming that other factors affecting amylose and ELC content were unchanged by the transgene expression, a single copy of the Wx^a gene and the resulting protein levels and enzymatic activity of GBSSI appeared to be sufficient to produce ~22% of apparent amylose, which is considered to be a threshold value in our experimental conditions. A highly positive relationship between the amounts of Wx proteins and ELC contents in starch has been reported by Inouchi et al. (2005). In this study such a correlation was not observed when amino acid substitution existed in the Wx^a gene of cv. Labelle, as described below. Other factors that could affect amylose synthesis are ADP-glucose levels (Fulton et al. 2002) and three-dimensional space allowance. A similar phenomenon was also observed in maize. Citing a work by Tsai (1974), Denyer et al. (2001) pointed out that in maize GBSSI activity was linearly proportional to *Wx* gene dosage in the range of zero (waxy) to three (wild type), but the amylose content of starches from the endosperms having two or three *Wx* genes was much lower than that expected from the gene dosage and GBSSI activity.

Table 4 Pasting properties of the starches from different rice cultivars (L, I and M) and transgenic rice lines (I26, M27 and M214)

Source	Pasting temperature (°C)	Viscosity (RVU)			Breakdown (RVU)	Setback (RVU)
		Max.	Min.	40°C		
L	75.5	242	165	230	77 (31.8)[a]	65
I	55.5	225	73	114	152 (67.6)	41
I26	64.3	95	83	152	12 (12.6)	69
M	59.7	193	82	128	111 (57.5)	46
M27	63.6	84	76	136	8 (9.5)	60
M214	64.8	105	84	166	21 (20.0)	82

Viscosity, breakdown and setback are expressed in Rapid Visco Analyzer Units (RVU). Breakdown and setback were calculated, respectively, as follows: maximum viscosity – minimum viscosity; viscosity at 40°C – minimum viscosity.
[a]Figures in parentheses are percentages of maximum viscosity.
L, cv. Labelle; I, cv. Iwaimochi; M, cv. Musashimochi.

Structures of amylose produced in transgenic lines

The amyloses synthesized in the transgenic lines exhibited a DP distribution slightly different from that of cv. Labelle. Although what determines the DP of amylose is not known, this finding implies a possible contribution of enzymatic properties of GBSSI to the size, and hence size distribution, of its products. The most striking difference was the increased molar ratio of branched molecules (Table 3). It may be due to the differences in properties of sets of branching enzymes between Labelle and the non-transgenic *wx* cultivars of Japonica rice. Another possible explanation is that the expressed GBSSI might have indirect effects on the formation of branches of amylose. In native starch granules amylose is thought to be present in the amorphous region of starch granules. Jane et al. (1992) showed that cross-linking between amylose and amylopectin occurs when native starch granules are subjected to reactions with cross-linking reagents. This observation suggests that three-dimensional spaces allowed for amylose and amylopectin synthesis, respectively, may not be strictly separated. Therefore, it is possible that in conditions of unusually high amounts of amylose, a portion of it could become more susceptible to the action of branching enzymes, probably at the interface between crystalline and amorphous regions.

Effect of the Wx^a transgene on starch pasting properties

Amylose content and ELC content are apparently the major factors affecting pasting properties of starch in the transgenic lines. The other factors affecting pasting properties, such as chain-length distribution of relatively short chains of amylopectin (Jane et al. 1999), effects of amylose–lipid complexes (Morrison et al. 1993) and bound phosphates (Blennow et al. 2001), are considered not significantly different in the transgenic lines compared with the *wx* cultivars.

In the initial phase of starch pasting, the primary effect of amylose is restriction of granule swelling, and restricted swelling has an influence on the subsequent pasting events, such as extent of swelling and disruption of swollen granules, which are measured as maximum viscosity and breakdown. These two properties were drastically decreased in the transgenic lines (Table 4) and were even much lower than for cv. Labelle. Since actual amylose contents were comparable among these starches, an effect of ELCs was thought to be involved. Although it is not well understood if the behavior and effect of ELCs is independent from, additive or synergistic to those of amylose, correlations between ELC content and some pasting properties have been reported. Inouchi and colleagues (Horibata et al. 2004, Inouchi et al. 2005) found that ELC content is positively and negatively correlated to setback and breakdown, respectively. Also Han and Hamaker (2001) reported a negative correlation of ELC content with breakdown. Therefore, it may be necessary to control ELC content, in addition to amylose content, in order to achieve the desired properties of starch.

What determines ELC formation on amylopectin molecules?

In cv. Labelle, which carries the Wx^a gene, amylopectin ELC was barely detected (Fig. 2) and the difference between the apparent and actual amylose contents was small. However, the transgenic lines contained high amounts of ELCs that caused much higher values for apparent amylose content than actual content (Table 1). These facts imply that the contribution of the GBSSI activity to the synthesis of ELC is different between Labelle and the transgenic lines. After surveying starches from 45 non-waxy rice cultivars, Horibata et al. (2004) proposed that rice cultivars can be classified into three groups, depending on their ELC content, high (13–16%), intermediate (5–7%) and low (<2%) ELC types.

To identify a possible cause of the differences in the amounts of ELCs, the amino acid sequence of GBSSI was

Table 5 *Wx* structure, expression, and ELC and amylose contents of some rice starches

Variety	5′ splice site of first intron	Relative amount of WX protein	Residue at position 224	Content (%)[a]		Accession No. of *Wx* gene
				ELC in amylopectin	Actual amylose in starch	
Wx^a						
IR36	/GT	High	Y	15	17	AB425323
Yumetoiro	/GT	High	Y	16	16	AB425326
WxR/*wx*	/GT	High	Y	8	15–16	AB425322
O. rufipogon	/GT	na	Y	na	na	DQ280673
Labelle	/GT	High	S	<1	13	AB425324
Hoshiyutaka	/GT	High	S	4	21	AB425325
Wx^b						
Nipponbare	/TT	Low	Y	<1	16	AK070431

[a]Some of the data were taken from the following references: cv. IR36, Takeda et al. (1987); cv. Yumetoiro, Horibata et al. (2004); cv. Hoshiyutaka, Mizukami et al. (1996); *WxR*/*wx*, cv. Labelle, and cv. Nipponbare, this study.
na, not available at this time.

compared between selected rice cultivars whose ELC contents are available in the literature. Differences in the *Wx* genes and GBSSI proteins sequences, and amylose (actual) and ELC content are summarized in Table 5. A well-characterized difference between the Wx^a and Wx^b genes is a nucleotide substitution in the 5′ splice site in the first intron of Wx^b. The mutation impairs splicing (Cai et al. 1998, Hirano et al. 1998, Isshiki et al. 1998, Larkin et al. 1999), resulting in a reduced amount of Wx protein in the endosperm of Wx^b (Sano et al. 1984). Carrying the Wx^a gene is apparently not sufficient to give a high ELC content since amylopectins with a low ELC content are found among cultivars carrying the Wx^a gene. Three cultivars with a high ELC content, *WxR*/*wx*, IR36 and Yumetoiro, have a Wx^a gene encoding a tyrosine residue at position 224 of GBSSI. The two cultivars with low ELC content, Labelle and Hoshiyutaka, were found to have a Y to S substitution at this position. We confirmed that none of the other single nucleotide polymorphisms (SNPs) generates amino acid substitutions in the entire *Wx* coding sequence (cds) of cvs. Labelle, and IR36 and *WxR*, by sequencing both the genomic and cDNA clones. Larkin and Park (2003) reported that the same amino acid substitution that resulted from the same SNP in exon 6 was associated with the apparent amylose content. One cultivar carrying the Wx^b gene, cv. Nipponbare, did not contain the Y224S substitution and had a low ELC content. *Oryza rufipogon*, probable progenitor of *O. sativa*, contains the Wx^a gene encoding Y at position 224, implying that rice cultivars with the Y224S mutation have been selected during domestication of rice, presumably for their moderate apparent amylose content and/or properties resulting from their low ELC content. Although only limited data were used, the comparison indicated that both the quantity and quality of GBSSI, namely above a certain amount of GBSSI having a Y residue at position 224, are required to form a relatively large amount of ELCs on amylopectin molecules.

Recently, Fujita et al. (2007) reported that high ELC-containing amylopectin was produced in rice endosperm of a starch synthase IIIa (SSIIIa)-deficient mutant that was derived from cv. Nipponbare, and the enriched ELC was explained by the increase of mRNA and protein levels of GBSSI. As shown in Table 5, cv. Nipponbare carries a *Wx* gene having the SNP directing Y residue at position 224 in the coding region, which is one of the two possible requirements for producing high ELC-containing amylopectin as discussed above. The other requirement, the expression level of GBSSI protein, is not satisfied in cv. Nipponbare, which carries the Wx^b gene. Therefore, we assume that in the SSIIIa-deficient mutant, increased expression of GBSSI with a Y residue at position 224 resulted in significant ELC formation, and an SSIIIa-deficient mutant having GBSSI with a Y224S mutation might show no changes in terms of ELC amount even when its GBSSI expression was increased.

Materials and Methods

Plant materials

All seeds were sown in early April, and the plants were cultivated at 28–32°C in a closed greenhouse under natural light. Flowering dates varied from mid-July to mid-August. Harvested seeds were used for starch purification. *Oryza sativa* L. cv. Iwaimochi (*wx*), cv. Musashimochi (*wx*) and cv. Labelle (Wx^a) were used for control experiments. High amylose transgenic lines carrying *WxR* genes were previously described in Itoh et al. (1997, 2003). The transgenic lines were produced by introduction of *WxR* into *wx* cultivars, Iwaimochi and Musashimochi, and the

transgenic lines I26 (Iwaimochi-26), M27 and M214 (Musashimochi-27 and -214) were used for this work.

Structural analysis of the Wx gene

Sequences of *Wx* cDNA of *O. rufipogon* and cv. Nipponbare were retrieved from GenBank, accession Nos. DQ280673 and AK070431, respectively. For the *Wx* sequences of cvs. IR36 and Labelle, and *WxR*, both genomic DNA and cDNA are sequenced, and the entire *Wx* cds and the region of the junction between the first exon and first intron were analyzed. For the *Wx* sequences of cvs. Hoshiyutaka and Yumetoiro, we partially sequenced both genomic DNA and cDNA, and analyzed the region of the junction between the first exon and the first intron, and the SNP for Y234 or S. The *Wx* gene structure of all cultivars was analyzed by GENENTYX (Software Development Co. Ltd., Tokyo, Japan). Cv. IR36 was provided by Professor T. Tanisaka (Kyoto University, Japan), and cultivars Yumetoiro and Hoshiyutaka were provided by Dr. N. Fujita (Akita Prefectural University, Japan).

Materials for starch structural analyses

Starch was prepared from rice endosperm by alkaline steeping in the cold. For Labelle and the transgenic lines, amylose and amylopectin were fractionated from defatted starch by the method of Lansky et al. (1949) with minor modifications (Takeda et al. 1986). The waxy starches were used without fractionation. The fractionated amyloses were confirmed to be free from amylopectin by gel permeation chromatography using Toyopearl HW-75F (Tosoh, Tokyo, Japan) (Takeda et al. 1984). ELCs were prepared by 1-butanol precipitation from debranched amylopectin from Labelle and the transgenic lines as reported previously (Hanashiro et al. 2005). Sweet potato β-amylase was obtained from Sigma Chemical Co. (St Louis MO, USA) and was purified by anion-exchange chromatography (Marshall and Whelan 1973) prior to use. *Pseudomonas* isoamylase was purchased from Hayashibara Biochemical Lab., Inc. (Okayama, Japan). 2-Aminopyridine, of a grade for fluorescent labeling, and sodium cyanoborohydride were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan) and Aldrich Chemical Co. Inc. (St Paul, WI, USA), respectively.

Analytical methods

The pasting properties of 9% (w/w) starch slurry were determined with a Rapid Visco Analyzer (RVA-3D, Newport Scientific, Narrabeen, Australia). Heating to 92.5°C and cooling to 40°C was performed at a rate of $3^{\circ}C\,min^{-1}$. The number-average degree of polymerization (DPn) and DP distribution of amylose and ELCs were determined by fluorescent labeling followed by HPSEC (Hanashiro and Takeda 1998). The molar fraction of branched and linear molecules in amylose and ELCs was determined by hydrolysis of labeled specimens with β-amylase and HPSEC of the hydrolyzate (Hanashiro et al. 2003). The number-average chain length (CLn) and CL distribution of starch and amylopectin were determined by fluorescent labeling followed by HPSEC (Hanashiro et al. 2002). CLn of amylose and ELCs were determined by the Smith degradation and fluorometric assay of glycerol (Hizukuri et al. 1981).

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