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Starch Physicochemical Properties and Their Associations with Microsatellite Alleles of Starch-Synthesizing Genes in a Rice RIL Population

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The physicochemical properties of starch, such as apparent amylose content, gelatinization temperature, and pasting viscosities, determine the eating, cooking, and processing qualities of various products of rice. A recombinant inbred line (RIL) population derived from the reciprocal cross of Lemont (a premium high-quality tropical *japonica* rice) and Jiayu 293 (a high-yield but low-quality *indica* rice) was used to test the association of microsatellite markers of starch-synthesizing genes with starch quality parameters. The results confirmed the association of *Wx* and *starch synthase I* (*SSI*) alleles with various starch properties measured in rice flour. However, the starch properties were not associated with the *starch branching enzyme 1* (*SBE 1*) gene alleles.

KEYWORDS: Microsatellite; paste viscosity; rice; simple sequence repeat; starch

INTRODUCTION

Rice is the staple food for half of the world's population. Eating, cooking, and processing qualities that affect cooked rice palatability and consumer acceptability have received increasing research attention in rice-producing countries in recent years. Rice chemists, in evaluating the quality of rice grain, have established that amylose content is among the most important determinants of eating and cooking quality (*1*). However, it is also agreed that there are differences among varieties that have similar amylose contents, such as in starch pasting viscosity (*1–3*). Genetic studies have increased dramatically our understanding of the genetic basis of such quality parameters (*2–12*). Rice breeders may take advantage of current knowledge derived from genetic studies, especially molecular genetic work, and integrate it into conventional breeding to develop new rice cultivars with improved grain quality (*13, 14*).

Starch comprises 90% of the total dry matter in the rice grain and thus plays an important role in grain quality. Through quantitative trait locus (QTL) mapping, apparent amylose content (AAC), pasting viscosity, and gel texture have been mapped at the *Wx* locus, which encodes granule-bound starch synthase (*2–8*). Gelatinization temperature (GT), thermal properties, and amylopectin structure have been mapped at the alkaline digestibility (*alk*) locus, which encodes starch synthase IIa (SSIIa) (*4–6, 8, 14*), or at the *Wx* locus (*7*), whereas retrogradation properties may be controlled by both the *Wx* and *alk* loci (*4, 9, 10*). Some microsatellite (also termed simple sequence repeat, SSR), single-nucleotide polymorphisms (SNPs) and sequence-tagged site (STS) markers for starch-synthesizing genes, including *Wx*, starch synthase I (*SSI*), *SSIIa*, starch-branching enzyme I (*SBE1*), and *SBE3*, have been developed (*3, 9, 11*). Association tests confirmed the results derived from QTL mapping (*9, 10*). However, marker-based selection for improvement of starch quality is still inadequately reported.

In this study, a recombinant inbred line (RIL) population derived from reciprocal crosses of Lemont (a premium high-quality tropical *japonica* rice) and Jiayu 293 (a high-yield but low-quality *indica* rice) was used to test association of SSR alleles of *Wx*, *SSI*, and *SBE1* genes with starch physicochemical properties, the results of which should prove useful for marker-assisted selection.

MATERIALS AND METHODS

Rice Materials. A reciprocal cross between Lemont (a premium high-quality *japonica* rice) and Jiayu 293 (a high-yield but low-quality *indica* rice) was made to study the genetic basis of grain quality in 1999. A total of 280 F_2 lines including 150 F_2 lines from a Lemont/Jiayu 293 cross and 130 F_2 lines from a Jiayu 293/Lemont cross were advanced to F_8 by single-seed descent. Finally, a RIL population consisting of a total of 270 lines was formed and used in this study. All of the lines were grown in the Zhejiang University farm from late May to October 2005. The leaf tissues of each line were harvested for DNA extraction, and the rice grains were harvested for analysis of physicochemical properties. After being air-dried and stored at room temperature for 3 months, the grain samples were milled to white rice

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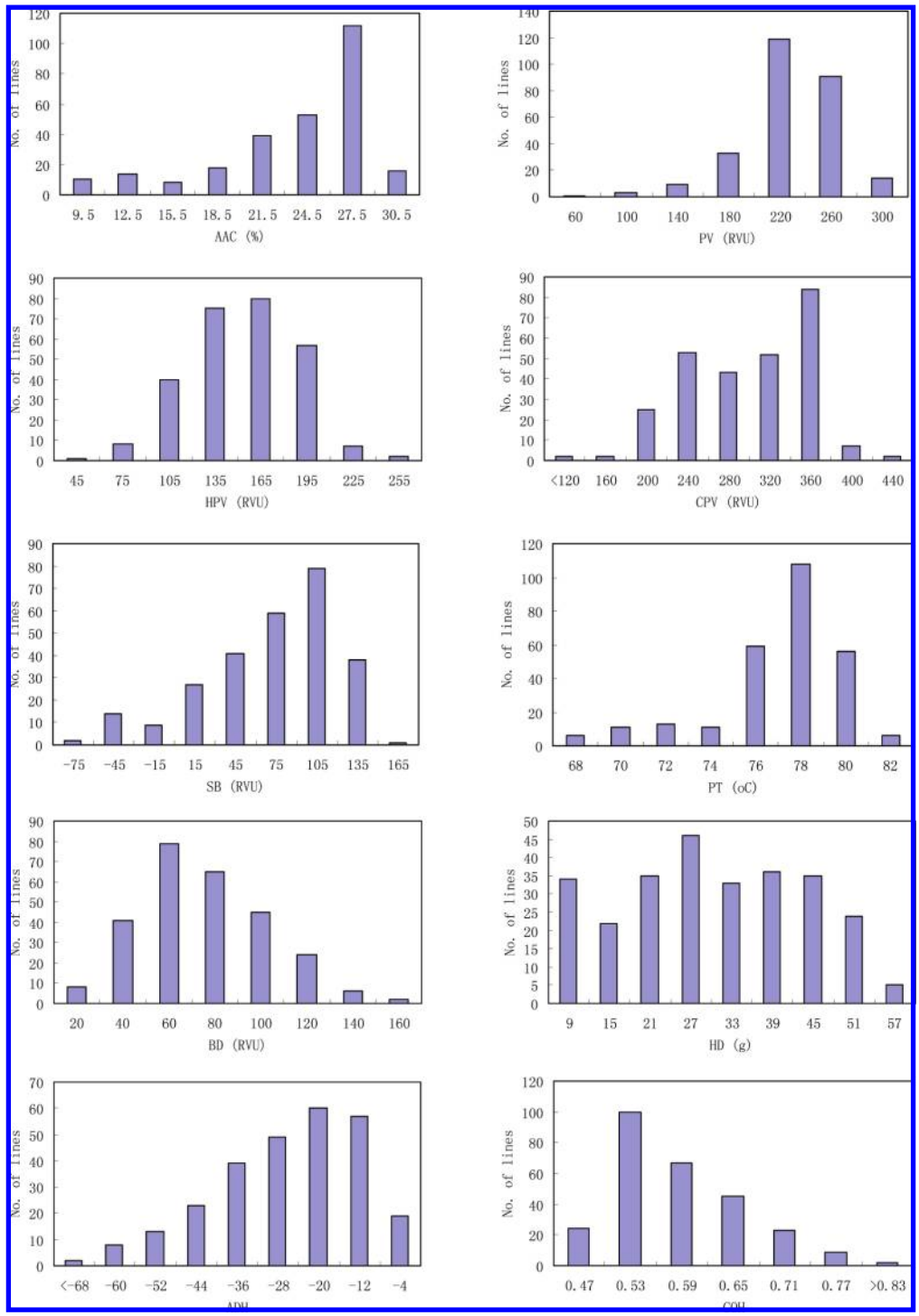

Figure 1. Distribution of starch physicochemical properties in the recombinant inbred line population.

using a Satake Rice Machine (Satake Corp.) and then ground to pass through a 100-mesh sieve on a Cyclone Sample Mill (UDY Corp., Fort Collins, CO).

Apparent Amylose Content (AAC). AAC was measured using the method of Perez and Juliano (*15*). Briefly, 100 mg of rice flour was put into a 100 mL volumetric flask, and 1 mL of 95% ethanol and 9 mL of 1 M sodium hydroxide were added; the contents were boiled for 8 min. After cooling for 1 h, the volume was made up with distilled water, 5 mL of the solution was put into a 100 mL volumetric flask, and 1 mL of 1 M acetic acid and 2 mL of iodine solution (0.2 g of iodine and 2.0 g of potassium iodide in a 100 mL aqueous solution) were added, and the volume was made up again with distilled water. The absorbance of the solution was measured at 620 mm with a spectrophotometer. A standard curve made simultaneously using rice samples of known amylose content was used to calculate the AAC of each sample.

Pasting Viscosity. Rice pasting properties were determined using a Rapid Visco Analyzer (RVA, model 3-D, Newport Scientific, Warriewood, Australia), using AACC International Standard Method AACC 61-02 (*16*). Flour (3 g, 12% mb) was mixed with 25 g of double-deionized water in the RVA sample can. The RVA was run using Thermocline for Windows software (version 1.2). A programmed heating and cooling cycle was used in which the samples were held at 50 °C for 1 min, heated to 95 °C in 3.8 min, held at 95 °C for 2.5 min before cooling to 50 °C in 3.8 min, and held at 50 °C for 1.4 min. The peak (PV), hot paste (holding; HPV), and cool paste (final) (CPV) viscosities and their derivative parameters breakdown (BD, = PV − HPV), setback (SB, = CPV − PV), and pasting temperature (PT) were recorded manually from the Thermocline for Windows software (version 1.2). The viscosity was measured in Rapid Visco Units (RVU).

Table 1. Starch Properties of Parents and Recombinant Inbred Lines (RIL)

	parents		RIL population				
parameter[a]	Jiayu 293	Lemont	mean ± SD	CV (%)	range	skewness	kurtosis
AAC (%)	27.2	21.0	23.8 ± 5.1	21.6	8.3–30.0	−1.2	0.8
PV (RVU)	246.7	219.7	228 ± 36.3	15.9	53.5–304.5	−1.0	2.5
HPV (RVU)	182.5	116.9	153.3 ± 35.1	22.9	35.9–262.9	−0.1	−0.2
CPV (RVU)	383.6	261.5	299.4 ± 59.3	19.8	77.1–441.7	−0.4	−0.3
BD (RVU)	64.2	102.8	74.7 ± 27.2	36.4	17.7–160.8	0.4	0.1
SB (RVU)	136.9	41.8	71.4 ± 48.5	68.0	−85.4 to 154.6	−0.9	0.2
PT (°C)	77.7	76.9	77.0 ± 2.8	3.7	67.6–82.0	−1.4	1.7
HD (g)	37.7	22.0	30.1 ± 13.2	43.7	6.5–56.5	0	−1.1
ADH (g·s)	−39.7	−27.1	−26.0 ± 14.3	55.1	−75.6 to −0.9	−0.7	0
COH	0.55	0.60	0.58 ± 0.07	105.01	0.45–0.87	0.89	0.54

[a] AAC, apparent amylose content; PV, peak viscosity; HPV, hot paste viscosity; CPV, cold paste viscosity; BD, breakdown viscosity; SB, setback viscosity; PT, pasting temperature; HD, gel hardness; ADH, gel adhesiveness; COH, gel cohesiveness. RVU, rapid visco unit.

Gel Texture. The resulting flour gels from RVA analysis were kept in the RVA canister, sealed with Parafilm and held at 4 °C for 24 h (*4, 5*). Texture profile analysis was carried out on a TA-XT2*i* Texture Analyzer (Texture Technologies Corp., Scarsdale, NY) equipped with the Texture Expert software program (version 5.16). A standard two-cycle program was used to compress the gels for a distance of 10 mm at a 4 mm/s speed using a 7 mm cylindrical probe with a flat end. Texture parameters of hardness (HD, g), adhesiveness (ADH, g·s), and cohesiveness (COH) were derived from the instrument software.

DNA Isolation. The genomic DNA was extracted from leaves of each line using the CTAB method (*17*).

Mitochondrial Marker. A mitochondrial marker (SSV039) (*18*) was used to trace the maternal parent of each line.

SSR Analysis. Primers used for amplifying microsatellites in the *Wx*, *SBE1*, and *SS1* genes followed Bao et al. (*3, 9*). The forward primers for *SSI* and *SBE1* SSRs were end-labeled with fluorescein Cy5 (Amersham Pharmacia Biotech). Each 20 μL amplification reaction consisted of 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 0.1% Triton X 100, 2 mM $MgCl_2$, 0.1 mM dNTPs, 200 nM primers, 1 unit of Taq polymerase, and 50 ng of genomic DNA. All amplifications were performed on a PTC-100 thermal cycler (MJ Research, Foster City, CA) under the following conditions: 5 min at 94 °C, followed by 45 s at 94 °C, 60 s at 55 °C, and 60 s at 72 °C for 35 cycles and 10 min at 72 °C for a final extension. The PCR product for *Wx* SSR was resolved by electrophoresis on a 3% agarose gel. The PCR products for *SSI* and *SBE1* SSR were mixed with an equal volume of formamide dye [98% formamide, 10 mM EDTA (pH 8.0), 0.1% bromophenol blue, and xylene cyanol]. After being denatured at 90 °C for 3 min and immediately chilled on ice, 5 μL of the sample was run through a 6% polyacrylamide gel for 5 h in an ALFexpress automated sequencer (Amersham Pharmacia Biotech.).

Statistical Analysis. All of the starch physicochemical property parameters were measured in duplicate. Analysis of variance (ANOVA) using general linear model was performed with the SAS System for Windows version 8 (SAS Institute Inc., Cary, NC) to test the associations between marker alleles and physicochemical data.

RESULTS

Mitochondrial Marker Analysis. The mitochondrial genome is maternally inherited. To check for mixing or mishandling, mitochondrial markers can be used to check the maternal status of each line. Lemont and Jiayu 293 had different alleles at the mitochondrial marker locus, and in the RIL populations, each line had an allele matching one of the parents. Finally, 146 lines from the Lemont/Jiayu 293 cross and 124 lines from the Jiayu 293/Lemont cross were confirmed after mitochondrial marker analysis to have the expected maternal allele.

Analysis of Starch Physicochemical Properties. As is common practice in large-scale genetic studies of rice starch, flour samples were usually used to infer starch properties. Significant differences in the starch properties, except for pasting temperature (PT), were observed between the two

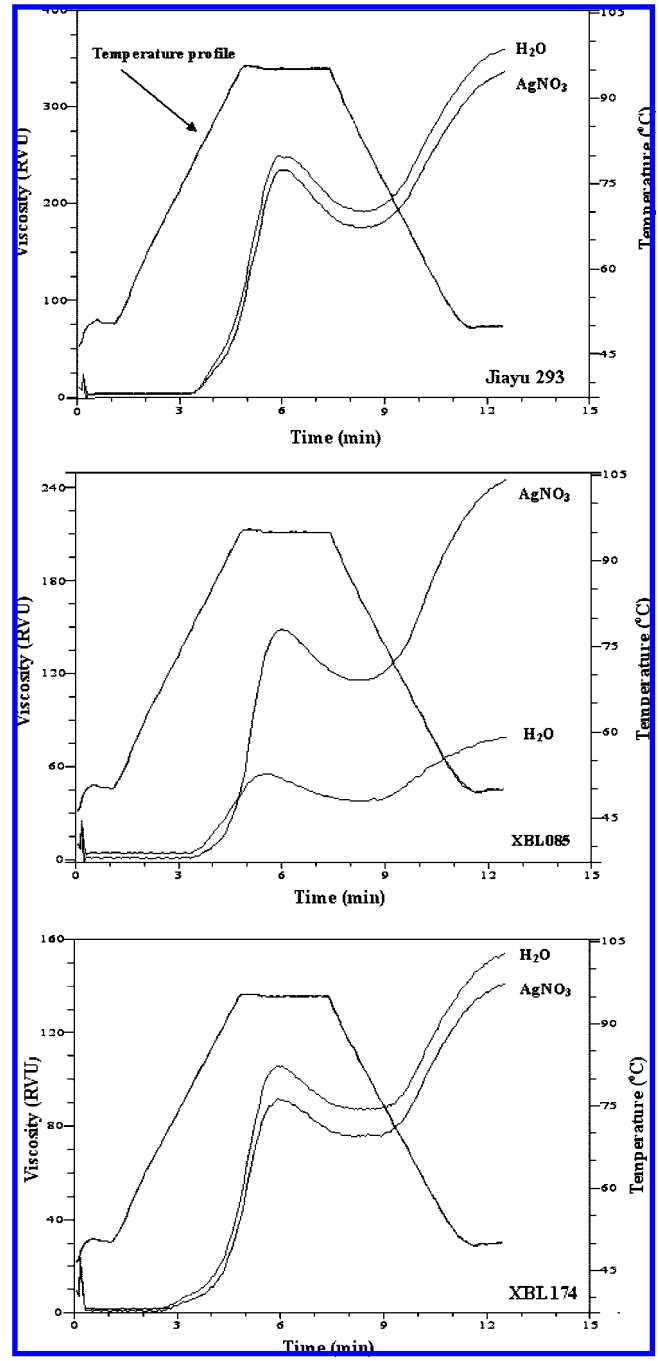

Figure 2. Response of parent (Jiayu 293) and low-viscosity lines to 0.5 mM $AgNO_3$ in the Rapid Visco Analyzer (RVA) analysis.

Table 2. Comparison of Starch Properties with Different Marker Groups

marker[a]	AAC	PV	HPV	CPV	BD	SB	PT	HD	ADH	COH
mtDNA[b]										
mt-J(123)	23.3	233.4	155.6	299.4	77.8	66.0	76.3	30.9	−28.4	0.58
mt-L (144)	24.2	226.3	153.3	298.5	73.0	76.8	77.5	30.0	−24.3	0.58
R^2[c]							0.048		0.021	
Wx										
$(CT)_{11}$(150)	27.2	236.4	176.2	341.2	60.1	104.8	76.6	39.7	−32.7	0.53
$(CT)_{20}$(105)	19.0	222.1	125.3	247.2	96.8	25.1	77.3	18.7	−17.4	0.64
R^2	0.624	0.047	0.556	0.668	0.445	0.636	0.016	0.585	0.282	0.558
SS1										
SSS-B (137)	26.0	234.1	167.8	326.2	66.3	92.1	76.7	36.2	−29.6	0.55
SSS-C (124)	21.5	225.6	140.6	274.9	85.0	49.4	77.2	24.6	−23.2	0.61
R^2	0.201		0.161	0.204	0.121	0.193		0.199	0.051	0.183
SBE1										
SBE-A (231)	23.7	230.7	155.8	302.3	74.9	71.6	76.9	30.5	−26.2	0.58
SBE-B (35)	24.3	221.0	145.1	295.1	75.9	74.2	77.3	29.6	−26.4	0.57
R^2										

[a] The value in parentheses is the total number of lines with this allele. See **Table 1** for abbreviations. [b] mt-J and mt-L stand for the same alleles as parent Jiayu 293 and Lemont, respectively. [c] R^2 is given only when the difference is significant ($p < 0.05$).

parents (**Table 1**). Wide segregation was found in the RIL population (**Table 1**; **Figure 1**). The mean of the RIL lines for ADH was slightly greater than that of both parents, but the mean of other traits was between the two parents (**Table 1**). The apparent amylose contents of Jiayu 293 and Lemont were 27.2 and 21.0%, respectively, but a few lines had AAC of <16%. Similarly, the PTs of both parents were similar (about 77 °C), but there were lines with PT at <72 °C and others at >80 °C (**Figure 1**). This indicates strong transgressive segregation in the population. This was also true for all other measured traits (**Figure 1**). A continuous distribution for each trait was observed in the RIL lines (**Figure 1**). The skewness value of AAC and PT and the kurtosis value of PV and PT were larger than 1, indicating that the distributions of AAC, PV, and PT were skewed, whereas the distributions of the other traits were normal (**Table 1**).

There were four lines with peak viscosity in water of <120 RVU (XBL85, 166, 174, and 230), which could have been due to high α-amylase activity. Because viscosity analysis could be used to indirectly estimate α-amylase activity (*19*), viscosities of these lines were measured again in 0.5 mM $AgNO_3$ solution. After measurement of the viscosity in the $AgNO_3$ solution, three lines (XBL85, 166, and 230) showed dramatically increased viscosities, whereas the increases in the viscosities of XBL174 and the parents were small (**Figure 2**). Thus, low viscosity in the three lines (XBL85, 230, and 166) was likely caused by high endogenous α-amylase activity, which may be derived from early ripening or spontaneous mutations.

Analysis of SSR Alleles. It is known that the SNP of *Wx* and *SSIIa* genes, sequence-tagged site (STS) of *SBE1*, are the same between parents Jiayu 293 and Lemont, whereas the SSRs of *Wx*, *SSI*, and *SBE 1* are different (*9*). Thus, we analyzed only the RIL population with these three SSR markers.

It is known that the Wx SSR allele of Jiayu 293 is $(CT)_{11}$, whereas that of Lemont is $(CT)_{20}$ (*3, 9*). Of the 270 inbred lines studied, 152 lines had the $(CT)_{11}$ allele and 106 had the $(CT)_{20}$ allele, whereas both $(CT)_{11}$ and $(CT)_{20}$ alleles were present in the remaining 12 lines.

It is known that the alleles of Jiayu 293 and Lemont at the *SSI* SSR locus are the SSS-B allele $[(AC)_3...TCT(TC)_6...(TC)_4C(ACC)_9]$ and SSS-C allele $[(AC)_3...TCT(TC)_6...(TC)_4C(ACC)_8]$, respectively (*3, 9*). Among the 270 lines in this study, 139 lines had the SSS-B allele and 125 lines had the SSS-C allele, whereas both SSS-B and SSS-C alleles were present in the remaining 6 lines.

It is also known that the *SBE1* SSR alleles of Jiayu 293 and Lemont are the SBE-A allele $[CTCTCGGGCGA...(CT)_{10}]$ and SBE-B allele $[CTCTCGGGCGA...(CT)_8]$, respectively (*3, 9*). The *SBEI* alleles showed a highly skewed segregation; of the 270 lines, 234 lines had the SBE-A allele, 35 lines had the SBE-B allele, and only 1 line had both the SBE-A and SBE-B alleles.

Association of SSR and Starch Properties. ANOVAs were performed to compare means of starch properties between different alleles of mtDNA and SSR markers of *Wx*, *SSI*, and *SBE1* genes (**Table 2**). Three lines with low viscosities and all of the heterozygous or polymorphic lines were excluded from the association test. Only for PT and ADH was there any significant difference between the two mtDNA marker groups (**Table 2**), indicating that maternal and/or cytoplasmic effects might affect these two traits (*20*). However, this mtDNA marker could explain little (4.8 and 2.1%) of the total variation in PT and ADH. Because for all other traits there was no difference between the two mtDNA groups, ANOVA was performed for the SSR markers with all of the RILs included. Means for all of the traits differed between the two *Wx* SSR alleles, indicating that the *Wx* gene was closely associated with these starch traits. The *Wx* locus could explain >44% of the total variation for AAC, HPV, CPV, BD, SB, HD, and COH and 28% of the total variation for ADH. Even though R^2 was significant for PV and PT ($P < 0.05$), the *Wx* locus explained only 4.7 and 1.6% of the total variation, respectively. *SSI* is closely linked with *Wx* (*21*), and only two traits (PV and PT) could not be differentiated by the two *SSI* groups. Nearly 20% of the total variation of AAC, HP, CPV, SB, HD, and COH could be explained by the *SSI* locus. The *SBEI* alleles showed highly distorted segregation, but none of the traits showed significant mean difference between the two *SBE1* groups (**Table 2**).

DISCUSSION

Rice genetics and breeding have entered a genomic era in which genetic variation can be explained by different kinds of markers and even single nucleotide differences of the genetic code. There are two main marker resources that can be used in breeding practice. The first is QTLs from a specific mapping population with two parents. This kind of marker is population-dependent. Thus, in a cross with two other parents, further verification is needed whether the QTLs are still related to the traits using the same mapping approaches. The second resource

is markers derived from specific genes. These markers are functional and may be invariably associated with specific traits. For example, the SSRs and SNPs from the *Wx* and *SSIIa* genes are responsible for amylose content and gelatinization temperature, respectively (*10, 11, 22*). However, the marker−trait association should be confirmed in the form of cosegregation in a segregating population. This kind of marker is population-independent; that is, markers can be used in any cross with any parents.

The SSRs examined in this study are markers of the latter type (*3, 9*). Whether they are truly associated with starch physicochemical properties was not investigated before. The present results indicated that the lines with *Wx* allele $(CT)_{11}$ had AAC, PV, HPV CPV, SB, and HD values significantly higher than those with allele $(CT)_{20}$, but their BD, PT, ADH, and COH values were significantly lower (**Table 2**). Bao et al. also found these differences between $(CT)_{11}$ and $(CT)_{20}$ in different rice germplasms except for PT, for which there was no difference (*9*). Thus, the present results from cosegregation analysis confirmed the association of *Wx* alleles with all of these starch physicochemical properties (*9*). This kind of association is also supported by results from QTL mapping studies (*2–7*). Thus, marker-assisted selection using *Wx* genic marker is appropriate to alter amylose content, pasting viscosity, and gel texture. Bao (*12*) studied the relationship between the *Wx* microsatellite alleles and starch quality parameters with 36 F_2 plants derived from the cross between Longtefu A [*Wx* allele $(CT)_{11}$] and 371 [*Wx* allele $(CT)_{18}$]. The results indicated that significant differences in AAC, gelatinization temperature (GT), gel consistency (GC), and starch paste viscosity parameters were found among different *Wx* alleles. Zhou et al. demonstrated that the *Wx* marker can be used to improve eating and cooking qualities of hybrid rice, such as Shanyou 63, a hybrid rice derived from Zhenshan 97A (a male-sterile line) and Minghui 63 (a restorer line) (*20*). Minghui 63 is a good-quality rice characterized as having medium AAC, soft GC, and high GT. Zhenshan 97B and Zhenshan 97A are poor-quality rices characterized by high AAC, hard GC, low GT, and chalky endosperm. The improved versions of Zhenshan 97B and Zhenshan 97A had quality similar to that of Minghui 63 by introgressing the *Wx* gene region from Minghui 63. Thus, the improved hybrid rice Shanyou 63 had the desired lower AAC and increased GC and GT, coupled with a reduced grain opacity (*20*).

Previous association testing indicated that starch synthase I (*SSI*) could differentiate starch quality into different groups (*9*). The SSS-B allele had higher mean HPV, CPV, SB, PT, and HD and lower BD, ADH, and COH than the SSS-C allele in nonwaxy rice (*9*). Even in waxy rice, the SSS-B allele still had higher HPV and CPV than the SSS-C allele (*3*). In the present study, all parameters except PV and PT had significant difference between the two *SSI* alleles (**Table 2**), thus confirming that *SSI* alleles are associated with most starch physicochemical properties. It should be mentioned that Larkin et al. (*23*) once suggested that this may primarily be a linkage effect because the *SSI* locus is only 5–10 cm away from *Wx* locus (*21*).

Previous study showed that the SBE-A group had higher AAC, PV, HPV, CPV, HD, and COH than the SBE-B group, but no difference in BD, SB, and PT in nonwaxy rice (*9*). In waxy rice, HPV and CPV in the SBE-A group were still higher than those in the SBE-B group (*3*). However, these differences were not observed in the present study because all of the starch physicochemical properties were the same between the two *SBE1* alleles in the RIL population (**Table 2**).

It seems puzzling that the pasting temperature still segregated in the RIL lines, although the parents, Jiayu 293 and Lemont, have the same SSIIa alleles and pasting temperature. This could be explained by other functional genes regulating gelatinization temperature, in addition to some microenvironmental effects. Shu et al. reported that a low gelatinization temperature mutant is not caused by the *SSIIa* gene, but by other genes closely linked to *Wx* (*24*). Because the mode of inheritance of gelatinization temperature is very complex (ref *20* and references cited therein), further analysis of the genetic control of the gelatinization temperature (or pasting temperature) is needed.

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