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Microsatellites in starch-synthesizing genes in relation to starch physicochemical properties in waxy rice (*Oryza sativa* L.)

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Abstract Rice starch is composed of amylose and amylopectin. Amylose content, an important determinant of rice starch quality, is primarily controlled by the *waxy* gene, encoding granule-bound starch synthase (GBSS). The starch branching enzyme (SBE) and soluble starch synthase (SSS) play major roles in the synthesis of amylopectin. Microsatellite polymorphisms in the three genes, the *wx* gene encoding granule-bound starch synthase I, the *SBE* gene encoding starch branching enzyme I and the *SSS* gene encoding soluble starch synthase I, were studied for 56 accessions of waxy rice (*Oryza sativa* L.). Four $(CT)_n$ microsatellite alleles, $(CT)_{16}$, $(CT)_{17}$, $(CT)_{18}$ and $(CT)_{19}$, at the *wx* locus were detected in this set of waxy rice, of which $(CT)_{17}$ was the most frequent. Three $(CT)_n$ microsatellite allele classes were found at the *SBE* locus, $(CT)_8$ or $(CT)_{10}$ together with an insertion sequence of CTCTCGGGCGA, and $(CT)_8$ alone without the insertion. There were multiple microsatellites clustered at the *SSS* locus. However, these alleles can also be grouped into three classes, i.e. the allele class *SSS*-A = $(AC)_2\ldots TCC(TC)_{11}\ldots(TC)_5C(ACC)_{11}$, the allele class *SSS*-B = $(AC)_3\ldots TCT(TC)_6\ldots(TC)_4C(ACC)_9$, and the allele class *SSS*-C = $(AC)_3\ldots TCT(TC)_6\ldots(TC)_4C(ACC)_8$. The analyses of starch physicochemical properties among different microsatellite genotypes indicated that the *waxy* rice group with the $(CT)_{19}$ allele, the *SBE*-A allele and the *SSS*-B allele was quite different from other groups. Nine out of 15 accessions with a high gelatinization temperature (GT) belonged to the *wx* $(CT)_{19}$ group, all of them belonged to the *SBE*-A group and 13 of them belonged to the *SSS*-B group. These microsatellites might be useful in marker-assisted breeding for the improvement of rice grain quality.

Keywords Microsatellite · Rice · Starch quality · Soluble starch synthase · Starch branching enzyme · *wx*

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Introduction

Rice eating and cooking quality are mainly influenced by the physical properties of its starch. Starch is composed of amylose and amylopectin, and the apparent amylose content (AAC) is recognized as one of the most important determinants of the eating and cooking quality of rice. Rice eating quality still differs among varieties with a similar AAC, which can be explained by differences in amylopectin structure (Reddy et al. 1993; Ong and Blanshard 1995a, b). Therefore, other testing methods, such as gel consistency and pasting viscosity, have been established in order to differentiate quality among those varieties with a similar AAC.

Biochemically, amylose content is controlled by the amount of granule-bound starch synthase (GBSS) (also known as waxy protein), the product of the *wx* gene, whereas the starch branching enzyme (SBE) and the soluble starch synthase (SSS) play major roles in the synthesis of amylopectin (Smith et al. 1997). Genetic studies have indicated that some starch quality parameters might be controlled by a few alleles (one to three) with major effects, and by one or more modifiers (Chang and Li 1991; He et al. 1999; Bao et al. 2000). This is especially true for AAC, which is consistent with the fact that GBSS is necessary for the synthesis of amylose in rice. However, for endosperm traits, the genetic basis for starch quality would be more complex because the traits might be affected by quantitative genes of the triploid endosperm, the cytoplasm and the maternal plant genome (Bao and Xia 1999; Bao et al. 2002a).

Using RFLP analysis, two alleles of the *wx* locus were identified (Sano et al. 1986). The allele wx^a is characteristic of *indica* rice, and wx^b is found mainly in *japonica* rice (Sano et al. 1986). The difference between the two alleles is considered by many researchers to be responsible for differences in the levels of the *wx* gene-product in the endosperm, and consequently the differences in AAC. Wang et al. (1995) observed that AAC and the level of waxy protein in 31 rice cultivars from China were correlated with the ability of the cultivars to excise the leader intron of the *wx* transcript. This observation has been confirmed later by many other researchers who found that low amylose cultivars had the sequence AGTTATA at the putative leader intron 5′ splice site, while all intermediate- and high- amylose cultivars had AGGTATA (Wang et al. 1995; Bligh et al. 1998; Cai et al. 1998; Isshiki et al. 1998). Ayres et al. (1997) found the G to T mutation could explain 79.7% of the variation in the AAC of the nonwaxy cultivars.

However, these two alleles are not adequate to explain all the observed variation in AAC among the commercial rice cultivars, because some *indica* rice has a lower AAC than *japonica* rice. On introducing the wx^b allele into an *indica* background, AAC is reduced to be lower than in the wx^b *japonica* background, suggesting the possible importance of modifiers that regulate expression of the *wx* gene (Mikami et al. 2000).

A polymorphic $(CT)_n$ microsatellite was identified in the 5′-untranslated region of the *wx* gene (Bligh et al. 1995). Ayres et al. (1997) and Shu et al. (1999) identified eight *wx* microsatellite alleles which together explained more than 82% of the variation in AAC of nonwaxy rice. This same microsatellite explained 88% of the variation in AAC of 198 U.S. cultivars and breeding lines grown in different locations (Bergman et al. 2001), further demonstrating its correlation with various classes of AAC in nonwaxy rice. This microsatellite can thus be used as a molecular marker by rice breeders to more rapidly develop cultivars with a desirable amylose content.

The above-mentioned research mainly involves nonwaxy rice. To-date, the allele frequencies of the *wx* $(CT)_n$ microsatellite in waxy rice, and their relationships with physicochemical parameters (other than AAC), have not been comprehensively studied. In nonwaxy rice, the role of amylopectin in eating and cooking quality is, at least in part, masked by amylose. But in waxy rice, which contains only amylopectin, the eating and cooking quality should be predominantly affected by the amylopectin properties. There are also microsatellites in the rice starch-branching enzyme-I gene (*SBE*) (Genbank accession no. D10838) and the soluble starch synthase-I gene (*SSS*) (Genbank accession no. D38221). It has not been determined whether these microsatellites are correlated with starch properties. The objective of the present study was to analyze microsatellite polymorphisms in the *wx*, *SBE* and *SSS* genes in waxy rice, and to clarify whether the alleles at each of the microsatellite loci could differentiate the starch quality groups.

Materials and methods

Rice materials

A total of 56 waxy rice accessions (varieties, landraces and breeding lines) were obtained from the rice germplasm center and rice breeding programs in China (Table 1). These accessions represent the provinces of Zhejiang, Guizhou, Henan, Hubei, Jiangsu, Shanxi, Yunnan, Hainan, Jiangxi and Hebei; and Accession 664 and Accession 682 were from Japan. Thirty four of the 56 accessions were *indica* rice and 22 were *japonica* rice. All accessions were planted in Hainan in late November, 1999, and harvested in late March, 2000. Ten accessions of nonwaxy rice were included as references (Table 1), some of which have known-*wx* $(CT)_n$ microsatellites.

DNA isolation

Five seeds of each accession were germinated for DNA extraction. Genomic DNA was extracted from fresh young leaves using the CTAB method (Doyle 1991).

Microsatellite analysis

The primers used for amplifying microsatellites in the *wx*, *SBE* and *SSS* genes are given in Table 2. The forward primers, 484, 486 and 488 were end-labeled with fluorescein Cy5 (Amersham Pharmacia Biotech.). Each 20-μl amplification reaction consisted of 10 mM of Tris-HCl (pH 9.0), 50 mM of KCl, 0.1% Triton ×-100, 2 mM of $MgCl_2$, 0.1 mM of dNTPs, 200 nM of primers, 0.5 units of *Taq* polymerase, and 20 ng of genomic DNA. All amplifications were performed on a PTC-100 thermal cycler (MJ Research, Inc.) 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 7 min at 72 °C for a final extension. The amplification products were mixed with an equal volume of formamide dye (98% formamide, 10 mM of 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 using an ALFexpress automated sequencer (Amersham Pharmacia Biotech.). A DNA ladder (ranging in length from 50 to 500 bp) labeled with Cy5 was used to determine the size of the microsatellite bands.

Sequence analysis

For sequence analysis of the *wx* microsatellite, a larger fragment was amplified using primers 484 and W2R (5′-tttccagcccaacaccttac-3′) (Ayres et al. 1997). For sequencing the *SBE* and *SSS* microsatellites, the same fragments amplified by primers 486/487 and 488/489 were used. The PCR product was purified with the High Pure PCR Product Purification Kit (Roche Diagnostics GmbH, Germany). DNA sequencing was performed on an ABI 377 automated sequencer following the manufacturer's instructions (Applied Biosystems, Inc.).

G/T polymorphism analysis

The AGGTATA/AGTTATA polymorphism at the putative 5′ leader intron splice site of the *wx* gene was detected by restriction endonuclease *Acc*I (BRL) according to Ayres et al. (1997).

Starch physicochemical properties

Starch thermal and retrogradation properties, pasting properties, and swelling volume had been analyzed and reported elsewhere (Bao et al. 2002b).

Table 1 The rice accessions and the microsatellites in the *wx*, *SBE* and *SSS* genes

Code	Accession	Subspecies[a]	*wx* $(CT)_n$	*wx* G/T[b]	*SBE*[c]	*SSS*[d]	GT[e]
Waxy rice							
1	Xinguangnuo	J	17	T	C	C*	L
2	Cungunuo	I	17	T	A	A*	L
3	Guinuo no. 1	I	17	T	A	A	L
4	Haocunuo	J	16	T	B	C	L
5	Huangjinnuo	J	16	T	C*	C	L
6	Jiainuo	I	17	T	A	A	L
7	Aiganyaxuenuo	I	17	T	A	A	L
8	Suyunuo	J	16	T	C	C	L
9	Accession 664	J	17	T	C	C	L
10	Accession 682	J	18	T	C	C	L
11	Chunjiangnuo no. 2	J	17	T	C	C	L
12	Guixiangsinuo	I	17	T	A	B*	H
13	Xiangnuo no. 4	J	18	T	B	C	L
14	Longqingzixiangnuo	J	17	T	B	C	L
15	148 nuo	I	17	T	A	A	L
16	Zhonghuazixiangnuo	I	17	T	A	C	L
17	Shao 9610	I	19*	T	A	B	H
18	Zaoxiangnuo	I	17	T	A	C	L
19	Biyunzaonuo	I	17	T	A*	B	H
20	Zhenongda 454	J	17	T	C*	C	L
21	Shaonuo 9617	J	17	T	B*	C	L
22	T1046	I	17	T	A	B	H
23	Zhenuo no. 2	J	17	T	C	C	L
24	Bing 9302	J	17	T	C	C	L
25	Zhuyunnuo	I	17	T	A	B	H
26	Shaonuo 9144	J	17	T	C	C	L
27	Chunjiangnuo 681	J	17	T	C	C	L
28	Chunjiangnuo no. 3	J	17	T	C	C	L
29	Bing 97252 nuo	J	17	T	C	C	L
30	Shaonuo 9714	J	17	T	C	C	L
31	Shaonuo 985	J	17	T	B	C	L
32	Shaonuo 928	J	17	T	C	C	L
33	Linxiangnuoxuan	J	19	T	B*	C	L
34	Lishuinuo	I	17	T	A	A	L
35	Cinuo 48	I	17	T	A	B	L
36	Dadongnuo	I	17	T	A	B	L
37	Shaximonuo	I	17	T	A	A	L
38	Dian 4	I	18	T	A	A	L
39	Yunanheixiannuo	I	17	T	A	A	L
40	Xiangnuo G982280	I	17	T	A	A	H
41	Ganxiangnuo	I	17	T	A	A	H
42	Yichunnuo 12	I	18	T	A	A	L
43	Zhongyu no. 8	I	17	T	A	A	L
44	Jiangxinuodao	I	18	T	A	A	L
45	Youzhizaoxiannuo	I	19	T	A	B	H
46	Zaojingnuo	I	19	T	A	B	H
47	Yangfunuo no. 3	I	17	T	A	B	L
48	PII 121	J	16*	T	B	C	L
49	Shao 9915	I	19*	T	A	B*	H
50	Yuenuo no. 1	I	19*	T	A	B*	H
51	Shao 9924	I	19	T	A	B	L
52	Shao 9929	I	19	T	A	B	H
53	Shao 9997	I	19	T	A	B	H
54	Shao 99102	I	19	T	A	B	H
55	Yuenuo 981	I	19	T	A	B	H
56	Hainannuodao	I	17	T	A	C	L
Nonwaxy rice							
57	Lemont	J	20*	G	B*	C*	MH
58	Azucena	J	19*	G	B*	C*	MH
59	Jingxi 17	J	18	T	C*	C*	L
60	Zhefu 49	I	18	T	A*	B	H
61	371	I	18	T	A	C	L
66	Minghui 63	I	18	T	A	C	H
62	IR64	I	17*	G	A*	C	MH
63	Xiushui 11	J	17*	T	C	C	L
64	Zhaiyeqing	I	11	G	A*	A*	H
65	Longtefu B	I	11*	G	A	A*	L
66	Zhefu 802	I	11	G	A*	B*	M

* Indicates the amplified fragment was sequenced

[a] I = *indica*; J = *japonica*

[b] T = AGTTATA; G: AGGTATA

[c] *SBE*-A: CTCTCGGGCGA...$(CT)_{10}$; *SBE*-B: CTCTCGGGCGA...$(CT)_8$; *SBE*-C: $(CT)_8$

[d] *SSS*-A: $(AC)_2$…TCC$(TC)_{11}$…$(TC)_5$ C$(ACC)_{11}$; *SSS*-B: $(AC)_3$…TCT$(TC)_6$…$(TC)_4$ C$(ACC)_9$; *SSS*-C: $(AC)_3$…TCT$(TC)_6$…$(TC)_4$ C$(ACC)_8$

[e] GT: gelatinization temperature of starch, L: low-GT; H: high-GT; M: medium-GT; MH: medium to high GT

Table 2 The primer sequences used to amplify microsatellite markers in rice starch-synthesizing genes

Gene	Forward primer (5′→3′)[a]	Reverse primer (5′→3′)	Accession	Reference
wx	484: ctttgtctatctcaagacac	485: ttgcagatgttcttcctgatg	X65183	Bligh et al. (1995); Ayres et al. (1997)
SBE	486: atttctttggccacaggcga	487: cccagattcggaacaagaac	D10838	Akagi et al. (1996)
SSS	488: gatccgtttttgctgtgccc	489: cctcctctccgccgatcctg	D38221	Temnykh et al. (2000)

[a] The forward primers, 484, 486 and 488, were 5′ end-labeled with Cy5 fluorescein

Statistical analysis

Analysis of variance (ANOVA) was performed with the SAS program version 6.04 (SAS Institute Inc., Cary, N.C., USA). Duncan's multiple range test was conducted for a comparison of means at $P < 0.05$.

Results

Microsatellites and G/T single-nucleotide polymorphism in the *wx* gene

Using the cultivars with known $(CT)_n$ repeat numbers as references, four classes of *wx* $(CT)_n$ microsatellites were identified in 56 waxy rices (Table 1, Fig. 1). The amplified products ranged from 120 to 126 bp in length and represented the $(CT)_n$ repeats of $(CT)_{16}$, $(CT)_{17}$, $(CT)_{18}$ and $(CT)_{19}$. The allele frequencies in the waxy rice samples were as follows: 36 accessions had the $(CT)_{17}$ allele, 11 accessions had the $(CT)_{19}$ allele, 4 accessions had the $(CT)_{16}$ allele, and the remaining 5 accessions had the $(CT)_{18}$ allele. The reference nonwaxy rice varieties also have the $(CT)_{11}$ and $(CT)_{20}$ alleles (Table 1, Fig. 1). The DNA sequencing data further confirmed all the $(CT)_n$ repeat numbers. All the waxy rice studied had the AGTTATA sequence at the putative leader intron 5′ splice site, while some nonwaxy rice had AGGTATA (Table 1). The nonwaxy variety IR64 with the $(CT)_{17}$ allele had the AGGTATA sequence, which was quite different from the others.

Microsatellites in the *SBE* and *SSS* genes

Three microsatellite alleles were amplified in a region of the *SBE* gene (Table 1, Fig. 1) in both the waxy and nonwaxy rice. DNA sequencing analysis showed that there were only two classes of $(CT)_n$ repeats, $(CT)_8$ and $(CT)_{10}$, at the locus (Fig. 2). However, an insertion sequence of 11 bp in length, CTCTCGGGCGA, was found just after the forward primer 486 in many accessions (Fig. 2). The three alleles detected in this study were classified as follows: the allele of CTCTCGGGCGA...$(CT)_{10}$ (*SBE*-A) had 206-bp in length, the allele CTCTCGGGCGA...$(CT)_8$ had 202-bp (*SBE*-B) and the allele $(CT)_8$ without the insertion sequence had 191-bp (*SBE*-C) (Table 1, Fig. 1). The allele $(CT)_{10}$ alone, without the insertion, was not detected. A total of 34 accessions of waxy rice had the *SBE*-A allele, indicating that

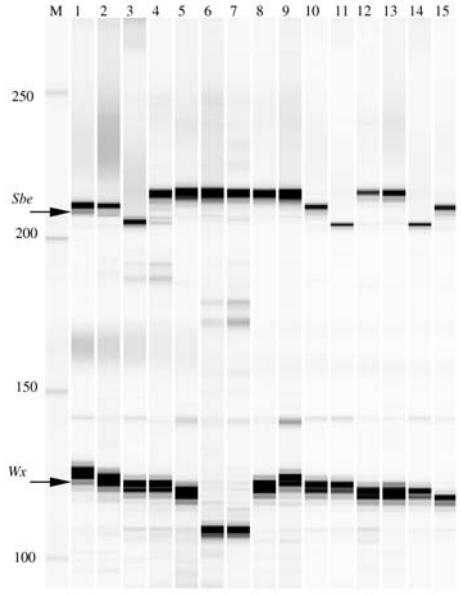

Fig. 1 Polymorphism of the microsatellites in the *SBE* (up) and *wx* (down) genes. *1* Lemont; *2* Azucena; *3* Jingxi 17; *4* 371; *5* IR64; *6* Zhaiyeqing 8; *7* Longtefu B; *8* Dian 4; *9* Shao 9915; *10* Xiangnuo no. 4; *11* Zaoshenghunuo; *12* Biyunzaonuo; *13* Zaoxiangnuo; *14* Xinguangnuo; *15* PII 121

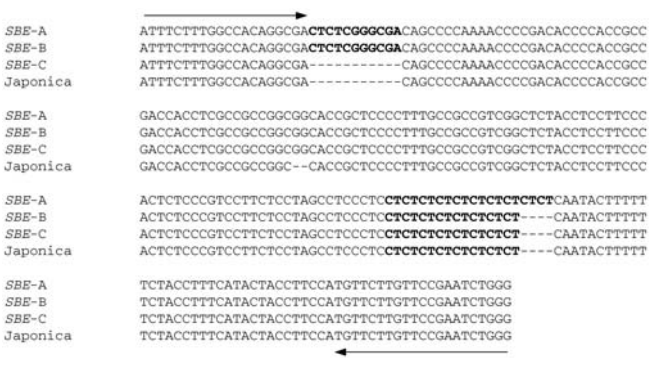

Fig. 2 Alignment of the microsatellite allele sequences of *SBE*. The microsatellites are in *bold*, the primers are *underlined*. The *japonica* rice represents the gene bank accession D10838

SBE-A was the most-frequent allele in the samples. Interestingly, all the 34 accessions are *indica* rice, suggesting that this microsatellite allele might be a subspecies specific marker. The remaining 7 and 15 accessions

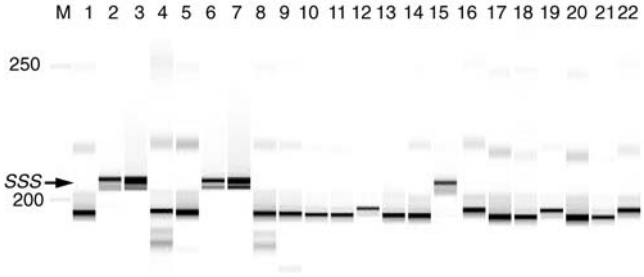

Fig. 3 Polymorphism of the microsatellite in the *SSS* gene. *1* Xinguangnuo; *2* Cungunuo; *3* Guinuo no.1; *4* Haocunuo; *5* Huangjinnuo; *6* Jiainuo; *7* Aiganyaxuenuo; *8* Suyuyuo; *9* Shenuo; *10* Zaoshenghunuo; *11* Chunjiangnuo no.2; *12* Guixiangsinuo; *13* Xiangnuo no.4; *14* Longqingzixiangnuo; *15* 148nuo; *16* Zhonghuazixiangnuo; *17* Shao 9610; *18* Zaoxiangnuo; *19* Biyunzaonuo; *20* Zhenongda 454; *21* Shaonuo 9617; *22* T1046; *23* Zhenuo no.2

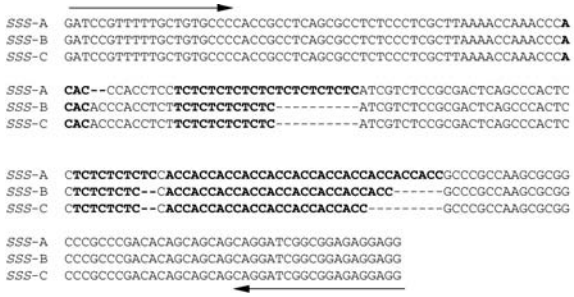

Fig. 4 Alignment of the three microsatellite allele sequences of *SSS*. The microsatellites are in *bold*; the primers are *underlined*

Table 3 Comparison of the mean starch physiochemical properties of four *wx* $(CT)_n$ classes in 56 waxy rice accessions. Means having a different letter are significantly different ($P < 0.05$). T_p: peak gelatinization temperature; ΔH_g: gelatinization enthalpy; $\Delta T_{1/2}$: width at half peak; ΔH_r: enthalpy of retrograded starch; FSV: flour swelling volume; PV: peak viscosity; HPV: hot paste viscosity; CPV: cool paste viscosity. RVU: Rapid Visco Analyser Units

wx	Number	T_p (°C)	ΔH_g (J/g)	$\Delta T_{1/2}$ (°C)	ΔH_r (J/g)	FSV (ml/g)	PV (RVU)	HPV (RVU)	CPV (RVU)
$(CT)_{16}$	4	68.7 b	8.0 b	8.5 a	1.5 b	17.5	125 b	73 c	94 c
$(CT)_{17}$	36	70.3 b	8.2 b	8.4 a	2.2 b	17.3	170 a	91 b	114 b
$(CT)_{18}$	5	68.3 b	8.3 b	8.5 a	1.4 b	17.4	179 a	89 b	112 b
$(CT)_{19}$	11	76.6 a	9.4 a	6.4 b	5.5 a	18.8	170 a	103 a	132 a

of waxy rice had the *SBE*-B and *SBE*-C alleles, respectively.

A total of three microsatellite alleles were found in a region of the *SSS* gene (Table 1, Fig. 3). Sequencing analysis showed that there were multiple microsatellites clustered in this region (Fig. 4). However, only three combinations of microsatellites were found as shown by the fragment size, i.e. $(AC)_2$…$TCC(TC)_{11}$…$(TC)_5$ $C(ACC)_{11}$ (218 bp, *SSS*-A), $(AC)_3$…$TCT(TC)_6$…$(TC)_4$ $C(ACC)_9$ (202 bp, *SSS*-B) and $(AC)_3$…$TCT(TC)_6$…$(TC)_4$ $C(ACC)_8$ (199-bp, *SSS*-C) (Fig. 3). Nearly half (27) of the waxy rice accessions had the *SSS*-C allele, 15 accessions had the *SSS*-B allele, and the remaining 14 accessions had the *SSS*-B allele.

The relationship between microsatellite classes and starch quality parameters

The relationship between the *wx* $(CT)_n$ microsatellites and starch physicochemical properties other than ACC has not been previously reported. No significant mean differences were found among the allele classes of *wx* $(CT)_{16}$, $(CT)_{17}$ and $(CT)_{18}$ in thermal properties, such as peak temperature (T_p), gelatinization enthalpy (ΔH_g), width at half peak ($\Delta T_{1/2}$), and retrogradation properties, such as the enthalpy of retrograded starch (ΔH_r), but the quality parameters of these three allele classes were significantly different from the $(CT)_{19}$ class. Nine out of the 11 accessions with $(CT)_{19}$ have a high gelatinization temperature (Table 1), therefore it is not surprising that all the thermal properties were higher in the $(CT)_{19}$ class than in the other classes. No significant differences were found in the pasting viscosity parameters, peak viscosity (PV), hot paste viscosity (HPV) and cool paste viscosity (CPV), between the $(CT)_{17}$ and $(CT)_{18}$ classes, but PV, HPV and CPV in the two classes were larger than the $(CT)_{16}$ class and smaller (except for PV) than the $(CT)_{19}$ class. There were no significant differences in flour swelling volume (FSV) among all the four *wx* $(CT)_n$ classes (Table 3).

Comparison of the starch physicochemical properties among the three microsatellite allele classes in the *SBE* gene showed that the *SBE-A* allele class was higher in T_p and ΔH_r than the *SBE*-B and *SBE*-C classes (Table 4). This might result from the fact that all the 15 high-GT accessions of waxy rice belonged to the *SBE*-A group (Table 1). The *SBE*-A and *SBE*-C groups had similar FSV values, which were higher than the *SBE*-B class. There was no significant difference in ΔH_g and PV among the three *SBE* classes.

No significant difference was found between the *SSS*-A and *SSS*-C classes in all starch-property parameters, but most parameters in these two classes were significantly less than in the *SSS*-B class (Table 5). These differences are apparently related to the GT values

Table 4 Comparison of the mean starch physiochemical properties of the three classes of *SBE* microsatellites in 56 waxy rice accessions. Means having a different letter are significantly different ($P < 0.05$)

Allele	Number	T_p (°C)	ΔH_g (J/g)	$\Delta T_{1/2}$ (°C)	ΔH_r (J/g)	FSV (ml/g)	PV (RVU)	HPV (RVU)	CPV (RVU)
SBE-A	34	72.6 a	8.5	7.5 b	3.6 a	18.3 a	171	95 a	121 a
SBE-B	7	69.1 b	8.3	8.3 ab	1.5 b	15.6 b	154	80 b	101 b
SBE-C	15	69.0 b	8.3	8.9 a	1.5 b	17.2 a	168	89 ab	112 ab

Table 5 Comparison of the mean starch physiochemical properties of the three classes of *SSS* microsatellites in 56 waxy rice accessions. Means having a different letter are significantly different ($P < 0.05$)

Allele	Number	T_p (°C)	ΔH_g (J/g)	$\Delta T_{1/2}$ (°C)	ΔH_r (J/g)	FSV (ml/g)	PV (RVU)	HPV (RVU)	CPV (RVU)
SSS-A	14	69.3 b	8.1 b	8.4 a	2.0 b	17.4 b	170	86 b	109 b
SSS-B	15	75.8 a	8.9 a	6.6 b	5.0 a	19.0 a	166	102 a	128 a
SSS-C	27	69.7 b	8.3 ab	8.6 a	1.8 b	17.0 b	168	89 b	113 b

among the waxy rice accessions, as the *SSS*-B allele class included 13 out of the 15 high-GT waxy rices (Table 1). No significant difference was found for PV among the three *SSS* classes.

Discussion

Previous studies have indicated that there are eight $(CT)_n$ microsatellite alleles located 55-bp upstream of the putative 5′-leader intron splice site in the *wx* gene (Bligh et al. 1995; Ayres et al. 1997; Shu et al. 1999). These alleles could explain a high percentage of variation in AAC of nonwaxy rice (Ayres et al. 1997; Shu et al. 1999; Bergman et al. 2001). However, it is unknown whether the same set of alleles are present in the waxy rice, because only a very limited number (three) of waxy rice accessions were included in the work of Ayres et al. (1997) and no waxy rice in others. The present study included 56 diverse accessions of waxy rice and clearly showed that only four $(CT)_n$ alleles were present, of which $(CT)_{17}$ was the most frequent (Table 1). All the waxy rice had the AGTTATA sequence at the putative leader intron 5′ splice site, whereas only some reference accessions of the nonwaxy rice had the AGGTATA sequence. Ayres et al. (1997) found that all the rice with AAC less than 18% had the sequence AGTTATA. Their data also showed that all the accessions with $(CT)_{17}$ or $(CT)_{18}$ had only the AGTTATA sequence, whereas the accessions with $(CT)_{19}$ had both AGTTATA and AGGTATA sequences. We found an exception in the nonwaxy accession IR64, which had $(CT)_{17}$, the AGGTATA sequence, and AAC of 19%. It is still unclear whether other alleles such as $(CT)_{11}$ and $(CT)_{20}$ at the microsatellite locus are present in waxy rice; if they are present, then whether the $(CT)_{11}$ or $(CT)_{20}$ waxy rice also have the AGTTATAC sequence, and whether other $(CT)_{17}$ or $(CT)_{18}$ waxy rice have the AGGTATA sequence is not clear. Further surveys of waxy rice germplasm will help address these questions.

The $(CT)_n$ microsatellite in the *SBE* gene is located in intron 2 (Genbank accession no. D10838). It was first revealed and mapped to rice chromosome 6 by Akagi et al. (1996), consistent with the locus of the original sequences of the starch-branching enzyme-I gene (Nakamura et al. 1994). However, the previous study only indicated that the microsatellite consists of $(CT)_n$. In this study, we found two CT repeat numbers, $(CT)_8$ and $(CT)_{10}$, which form three microsatellite alleles in association with an insertion sequence of 11 bp. We also found that the *SBE*-A allele is unique to *indica* rice, and the *SBE*-B and *SBE*-C alleles are unique to *japonica* rice. This allelic association with subspecies might result from selection in breeding history rather than from differentiation between *indica* and *japonica* subspecies. Thus, further studies of other diverse samples of the two subspecies are needed to confirm whether this microsatellite is truly subspecies specific.

The microsatellite in the *SSS* gene is located in the untranslated region just upstream of the transcription start site (Genbank accession no. D16202). It was first reported by Temnykh et al. (2000) as a $(ACC)_n$ microsatellite. Sequencing analysis in the present study revealed the presence of multiple classes of microsatellites clustered in the amplified region (Fig. 3). According to the band patterns and DNA sequencing data, we identified three composite microsatellite classes. Similar to the *SBE* microsatellite, there appears to be a subspecies-associated allelic distribution, as all the *japonica* rice had only the allele *SSS*-C, whereas *indica* rice had all three alleles (Table 1, Figs. 3 and 4). Again, this allelic distribution needs further verification. We noted the presence of a $(ACC)_7$ allele in a rice accession in the study of Temnykh et al. (2000), and the Genbank accession AF165890 had a $(ACC)_{20}$ allele at the same region of *SSS*. Thus it is apparently too early to draw conclusions on the allelic distribution of the microsatellite in this gene.

The relationships between the microsatellite classes and starch quality parameters showed that the rice geno-

types with $(CT)_{19}$, *SBE*-A and *SSS*-B allelic combinations were significantly different from the other groups. With one exception (accession Shao 9924, Table 1), this allelic combination is associated with high GT in waxy rice. Especially noticeable is the association between the allele *SBE*-A and gelatinization behavior in waxy rice, as all 15 high-GT waxy rice accessions belonged to the allele *SBE*-A class. However, not all waxy accessions with *SBE*-A had high GT, showing that the relationships between the microsatellite alleles and starch physicochemical properties are complex. Although all waxy accessions with *SSS*-C were associated with low GT, those nonwaxy rice accessions with *SSS*-C, such as Lemont, Azucena and Minghui 63, could have medium to high GT values (Table 1). It is thus necessary to further study these relationships by using near-isogenic lines that vary in both GT and microsatellite alleles in the same genetic background.

The question arises as to why and how the microsatellites in starch-synthesizing genes relate to the starch physical properties. Although there is no evidence indicating that the microsatellites themselves are directly responsible for the differences in starch physical properties, there is no doubt that GBSS, SBE-I and SSS-I, the products of the *wx*, *SBE* and *SSS* genes, are participating in starch synthesis (Smith et al. 1997). For example, the null mutations in the *wx* gene result in amylose-free starch in the endosperm of waxy rice. A single G to T mutation at the 5′ splice junction of the first intron in the *wx* gene reduces the efficiency of GBSS pre-mRNA processing and hence the level of spliced RNA (Wang et al. 1995; Bligh et al. 1998; Cai et al. 1998; Isshiki et al. 1998). The G/T polymorphism in nonwaxy rice is directly related to AAC. All the cultivars with 18% or less amylose were shown to have the sequence AGTTATA at the putative leader intron 5′ splice site, while all cultivars with a higher proportion of amylose had AGGTATA (Bligh et al. 1995, 1998). This single base change is considered to be responsible for the quantitative difference in AAC resulting from an altered regulation of the *wx* gene at the post-transcriptional level (Hirano et al. 1998).

More recent studies have found that mutations at other loci, such as *du-1* and *du-2*, also cause a reduced amylose content through their effects on the splicing of the waxy pre-mRNA (Isshiki et al. 2000), and that complete absence of amylose or its reduction was observed in transgenic rice whose wild-type *wx* gene expression was suppressed by antisense genes (Terada et al. 2000). These studies indicate that a variety of mechanisms are responsible for the waxy expression, including transcriptional and post-transcriptional processing, and translational regulation. So far there is no evidence that the microsatellite variation in the *wx* gene is a causal factor of the waxy expression.

Although it is still unknown whether the $(CT)_n$ microsatellites in the *wx* gene play any role in transcription and/or translation, its tight linkage with the G/T polymorphism renders it valuable as a molecular marker for the quantitative trait of amylose content in nonwaxy rice. These $(CT)_n$ microsatellite alleles explained 82–88% of the variation in AAC of nonwaxy rice (Ayres et al. 1997; Shu et al. 1999; Bergman et al. 2001), and have been used as a molecular marker to decrease the development time for the rice cultivars Cadet and Jacinto by several years (Bergman et al. 2001). However, in waxy rice, all having the AGTTATA mutation and lacking in amylose, the utility of this microsatellite as a molecular marker needs to be explored. The findings of this study suggest that it can potentially serve as a molecular marker for other starch qualities of waxy rice, such as GT-related thermal properties as well as some of the pasting viscosity parameters.

Similarly, the microsatellites in the *SBE* and *SSS* genes may also be useful as molecular markers for some of the starch quality parameters, such as GT and GT-related thermal properties, as well as some of the pasting viscosity parameters. Further research is needed to investigate whether these microsatellites are directly related to the quality traits, or the detected relations between the microsatellites and some of the starch quality parameters are due to linkages between the microsatellites and some functional polymorphisms in the genes affecting starch quality traits. A recent study by Umemoto et al. (2002) revealed that the *alk*, *gel*(*t*), *acl*(*t*) and *SSIIa* genes, responsible for the difference in amylopectin-related physicochemical properties of starch, all map to the same locus on chromosome 6 in the rice genome. This finding suggests that *alk*, *gel*(*t*), *acl*(*t*) and *SSIIa* are likely alleles of the same gene. Other genes that mapped to the same chromosome 6 include *wx*, *SS1* of *SSS* and *BEI* of *SBE*, although their respective locations are far from the *alk* gene (see Fig. 3 in Umemoto et al. 2002 and references therein). The *SS1* (= *SSS1*) and the *wx* gene were previously shown to be located close to each other (approximate map distance 5 centimorgans), whereas *alk*, a gene controlling the alkali spreading value and affecting gelatinization behavior, is more distant from *wx* and closer to the centromere (Tanaka et al. 1995). However, other genes may play additional roles in influencing the physicochemical behavior of rice starch (He et al. 1999; Umemoto et al. 2002), and the physical distances of the genes may not correspond to their genetic map distances as measured by actual recombination frequencies between the loci. For example, He et al. (1999) detected two loci controlling alkali disintegration and mapped one with a major effect at the *alk* locus. In contrast, Tan et al. (1999) mapped a single gene for the trait at the *wx* locus. The linkages or interactions among these genes may provide some insight into the complex relationships among the microsatellites in the *wx, SSS* and *SBE* genes, and some of the physicochemical properties of starch.

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