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ORIGINAL PAPER

Microsatellites, single nucleotide polymorphisms and a sequence tagged site in starch-synthesizing genes in relation to starch physicochemical properties in nonwaxy rice (*Oryza sativa* L.)

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Abstract Starch characteristics determine the quality of various products of rice, e.g., eating, cooking and processing qualities. Our previous study indicated that molecular markers inside or close to starch synthesizing genes can differentiate the starch properties of 56 waxy rices. Here we report microsatellite (or simple sequence repeat, SSR) polymorphism in the *Waxy* (*Wx*) gene, soluble starch synthase I gene (*SSI*) and starch branching enzyme 1 gene (*SBE1*), single nucleotide polymorphism (SNP) in *Wx* and starch branching enzyme 3 gene (*SBE3*), and a sequence tagged site (STS) in starch branching enzyme 1 gene (*SBE1*) among 499 nonwaxy rice samples and their relationships with starch physicochemical properties. The nonwaxy rice samples consist of landraces (n = 172) obtained from germplasm centers and cultivars and breeding lines (n = 327) obtained from various breeding programs. Ten $(CT)_n$ microsatellite alleles, $(CT)_8$, $(CT)_{10}$, $(CT)_{11}$, $(CT)_{12}$, $(CT)_{17}$, $(CT)_{18}$, $(CT)_{19}$, $(CT)_{20}$, $(CT)_{21}$, and $(CT)_{22}$, were found at the *Wx* locus, of which $(CT)_{11}$ was the most frequent, and $(CT)_{12}$, $(CT)_{21}$ and $(CT)_{22}$ were identified for the first time. Four $(CT)_n$ microsatellite alleles were found at the *SBE1* locus, $(CT)_8$, $(CT)_9$, and $(CT)_{10}$ together with an insertion sequence of CTCTCGGGCGA, and $(CT)_8$ alone without the insertion, of which $(CT)_9$ and the insertion was a new allele identified in only one rice, IR1552. Multiple microsatellites clustered at the *SS1* locus, and in addition to the three alleles previously detected (*SSS*-A = $(AC)_2$...TCC$(TC)_{11}$...$(TC)_5$ C$(ACC)_{11}$, *SSS*-B = $(AC)_3$...TCT$(TC)_6$...$(TC)_4$C$(ACC)_9$, and *SSS*-C = $(AC)_3$...TCT$(TC)_6$...$(TC)_4$C$(ACC)_8$), one new allele (*SSS*-D = $(AC)_2$...TCC$(TC)_{10}$...$(TC)_4$-C$(ACC)_9$) was found. Analysis of the starch physicochemical properties of the samples with different microsatellites, SNPs and STS groups indicated that these molecular markers can differentiate almost all the physicochemical properties examined, e.g., apparent amylose content (AAC), pasting viscosity characteristics, and gel textural properties. *Wx* SSR and *Wx* SNP alone explained more variations for all physicochemical properties than the other molecular markers. The total six markers could explain 92.2, 81 and 86% of total variation of AAC, gel hardness (HD), and gel cohesiveness (COH), respectively, and they could explain more than 40% of the total variation of hot paste viscosity (HPV), cool paste viscosity (CPV), breakdown viscosity (BD), setback viscosity (SB) and gel adhesiveness (ADH). However, only 29% of the total variation of peak viscosity (PV) and 37% of pasting temperature (PT) could be explained by all

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the molecular markers. Some of these markers can differentiate the starch physicochemical properties among the rice samples with the same *Wx* allele, indicating that the variation within *Wx* allele classes can be explained by other starch synthesizing genes. These SSRs, SNPs and STS are useful in marker-assisted breeding for the improvement of starch quality of rice.

Introduction

Rice serves as a staple food for about half of the world's people. New varieties with high yield potential, high quality and high resistance to biotic and abiotic stresses are bred and released continuously in order to meet the demand for more food arising from rapid human population growth and concurrent decrease in arable land. Improvement of rice quality is among the most important aims in current breeding programs, especially eating and cooking quality because most rice is consumed cooked. Processing quality has also received increasing attention because some rice has been used for specific food production, e.g., baby food, breakfast cereals, crackers, candies, noodles, unleavened breads and snack food (Bao and Bergman 2004; Juliano 1998). Rice eating, cooking and processing qualities are mainly influenced by physicochemical properties of its starch, which accounts for about 90% of milled rice. Starch is composed of amylose and amylopectin, and apparent amylose content (AAC) has been well recognized as one of the most important determinants of various rice products (Juliano 1998). However, rice eating and textural qualities still differ among varieties with similar AAC, which can be explained by differences in amylopectin structure (Reddy et al. 1993; Ong and Blanshard 1995a, b) and other factors, such as lipid and protein (Baxter et al. 2004). Thus, other testing methods, such as gel consistency and pasting viscosity, have been established in order to differentiate quality among those with similar AAC.

Biochemically, amylose synthesis requires granule-bound starch synthase (GBSS), the product of the *Wx* gene, whereas starch branching enzymes, starch synthases and starch debranching enzyme play major roles in the synthesis of amylopectin (Nakamura 2002, Satoh et al. 2003; Tomlinson and Denyer 2003). These starch-synthesizing genes may contribute to variation in starch physicochemical properties because they affect the amount and structure of amylose and amylopectin in rice grain. Genetic studies indicated that some starch quality parameters, such as AAC, gelatinization temperature, gel consistency and RVA pasting viscosity, might be controlled by one to three genes with major effects and one or more modifiers (see Chang and Li 1991; He et al. 1999; Bao et al. 2000; Bao et al. 2004; Wan et al. 2004; Fan et al. 2005). This is especially true for AAC, consistent with the fact that amylose production in rice is controlled by GBSS. The major genes for eating, cooking and textural qualities, such as *Wx* and *alk* (or *starch synthase IIa*, *SSIIa*), were confirmed by quantitative trait locus (QTL) mapping (He et al. 1999; Bao et al. 2000; Umemoto et al. 2002; Wan et al. 2004; Fan et al. 2005). However, the genetic basis for starch quality could be more complex for endosperm traits, because the traits might be affected by quantitative genes of triploid endosperm, cytoplasm and maternal plant genome (Bao et al. 2002a). Epistatic effects, i.e., interactions between genes, are also involved in controlling the eating and cooking quality traits (Fan et al. 2005).

A functional molecular marker is a DNA marker derived from a functionally characterized sequence motif, which is superior to random DNA markers owing to its complete linkage with the target gene (Andersen and Lübberstedt 2003). Two functional markers in the *Wx* gene, a $(CT)_n$ microsatellite (or SSR) and a G/T single nucleotide polymorphism (SNP), have been well characterized with different alleles differing in AAC (Bligh et al. 1995, Ayres et al. 1997; Shu et al. 1999). Recently, more gene tagged markers have been developed from starch synthesizing genes (Bao et al. 2002b; Han et al. 2004; Larkin and Park 2003; Larkin et al 2003; Oliver et al. 2002; Whitelaw et al. 2002). Bao et al. (2002b) found microsatellites in the *starch branching enzyme 1* (*SBE1*) and *starch synthase 1* (*SS1*) genes and Han et al. (2004) developed SNP and sequence tagged site (STS) markers in *SBE1* and *SBE3* genes for genotyping waxy rice, and the findings in these studies indicated that different alleles of these genes contribute to the variations in pasting viscosity characteristics and thermal properties in waxy rice. Larkin and Park (2003) discovered two SNPs in the exons of *Wx* that resulted in amino acid substitutions, and these SNPs were found to associate with differences in AAC and viscosity characteristics of 35 rice cultivars. Larkin et al. (2003) also discovered insertion/deletion markers for *SBE1*, *SBE3* and starch debranching enzyme gene, but they could not establish a relationship between the marker alleles and the variation in AAC and paste viscosity characteristics in a segregating population. Significant progress has been

achieved in developing functional markers for starch synthesizing genes, but systematic genotyping of rice materials is still needed to determine how many alleles exist at each locus and their relationships with starch physicochemical properties.

The objectives of this study are to detect polymorphisms in starch synthesizing genes among diverse landraces, cultivars and breeding lines of nonwaxy rice (*Oryza sativa* L.) and to determine their relationships with starch physicochemical properties. Specifically, we aim to clarify whether microsatellites of the *Wx*, *SBE1*, and *SS1*, SNPs of *Wx* and *SBE3*, and a STS of *SBE1* could differentiate these rices into starch physicochemical quality groups.

Materials and methods

Rice materials

We initially collected 577 rice accessions (coded from BP001-BP577), mostly from China (germplasm center and various rice breeding programs) and five of the accessions (BP035-BP040) from USDA-ARS, Rice Research Unit, USA. All the rice materials were planted in the winter season in Hainan (18°N), China, in November 2003. A total of 516 accessions with sufficient seeds were harvested for analysis of physicochemical properties (Bao et al. 2006a). Of these samples, 17 were identified as waxy rice according to the phenotype of chalky endosperm and measurement of apparent amylose content (Bao et al. 2006a). Because the microsatellites of waxy rice had been reported before (Bao et al. 2002b), we only focused on the nonwaxy rice ($n = 499$) including 172 landraces (the landrace set) and 327 cultivars and breeding lines (the breeding line set) in this study (see Appendix 1). A Dongxiang wild rice (*Oryza rufipogon*; BP580) was also included for marker genotyping.

DNA isolation

Rice seeds were germinated and genomic DNA was extracted from five seedlings of each rice accession using the CTAB method (Doyle 1991).

Microsatellite analysis

The primers used for amplifying microsatellites in the *Wx*, *SBE1* and *SS1* genes are given in Table 1. 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 Tris–HCl (pH 9.0), 50 mM KCl, 0.1% Triton × 100, 2 mM $MgCl_2$, 0.1 mM dNTPs, 200 nM primers, 0.5 unit 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 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.). The PCR products from rice samples with known microsatellite alleles were used as references.

SNP and STS analysis

The AGGTATAC/AGTTATAC polymorphism at the putative 5′ leader intron splice site of *Wx* gene was detected using restriction endonuclease *Acc*I (BRL) according to Ayres et al. (1997). Two fragments designated a G SNP, whereas no digestion indicated a T SNP. The ACTAGT/ACTACT SNPs at

Table 1 The primer sequences used to amplify SSR, SNP and STS markers in rice starch synthesizing genes

Gene (marker)	Forward primer (5′ → 3′)[a]	Reverse primer (5′ → 3′)	Reference
Wx (SSR)	484: ctttgtctatctcaagacac	485: ttgcagatgttcttcctgatg	Ayres et al. (1997)
Wx (SNP)	484: ctttgtctatctcaagacac	W2R: tttccagcccaacaccttac	Ayres et al. (1997)
SS1 (SSR)	488: gatccgtttttgctgtgccc	489: cctcctctccgccgatcctg	Bao et al. (2002b) Larkin et al. (2003)
SBE1(SSR)	486: atttctttggccacaggcga	487: cccagattcggaacaagaac	Akagi et al. (1996) Bao et al. (2002b)
SBE1(STS)	490: gagttgagttgcgtcagatc	491: aatgaggttgcttgctgctg	Han et al. (2004)
SBE3 (SNP)	492: gtcttggactcagatgctggactc	493: atgtataactggcagttcgaacgg	Han et al. (2004)

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

the 3′ untranslated region of *SBE3* was detected using restriction endonuclease *SpeI* according to Han et al. (2004). Two fragments (215 and 295 bp) designated a G SNP, whereas no digestion indicated a C SNP. A transposon insert at 335 bp upstream of the *SBE1* start codon was used to develop an STS marker, the expected size of PCR product is 882 bp for transposon insertion STS, whereas 547 bp for no insertion STS (Han et al. 2004). The primer sequences used to amplify these SNP and STS markers are given in Table 1.

Sequence analysis

For sequence analysis of the *Wx* microsatellite, a larger fragment was amplified using primers 484 and W2R (Ayres et al. 1997). For sequence analysis of the *SBE1* and *SS1* microsatellites, the same fragments amplified by primers 486/487 and 488/489 were used. The PCR product was purified with High Pure PCR Product Purification Kit (Roche Diagnostics GmbH, Germany). DNA sequencing was performed on an ABI 3100 automated sequencer following the manufacturer's instructions (Applied Biosystems, Inc.).

Starch physicochemical properties

After being air-dried and stored at room temperature for three months, all rice samples were milled to white rice using a Satake Rice Machine (Satake Corporation, Japan), and then ground to pass through a 100-mesh sieve on a Cyclone Sample Mill (UDY Corporation, Fort Collins, Colorado, USA). The flour apparent amylose content, pasting properties, and gel texture were determined (see Bao et al. 2006a) and the data were used in this study for marker association analysis.

Statistical analysis

The polymorphism information content (PIC) for each marker was calculated as described by Cho et al. (2000). Analysis of variance (ANOVA) was performed using the SAS System for Windows version 8 (SAS Institute Inc., Cary, NC, USA). Duncan's multiple range test was conducted for comparison of means at $P < 0.05$. General linear model was used in the analysis of the associations between marker alleles and different starch physicochemical data, which was performed using TASSEL (Trait analysis by association, evolution and linkage) software (http://www.maizegenetics.net/bioinformatics/tasselindex.htm).

Results

Microsatellites and G/T SNP in *Wx* gene

Using cultivars with known $(CT)_n$ repeat numbers as references, a total of 10 classes of *Wx* $(CT)_n$ microsatellites were identified in 499 nonwaxy rices (Table 1). The amplified products ranged from 106 to 132 bp in length and represented the $(CT)_n$ repeats of $(CT)_8$, $(CT)_{10}$, $(CT)_{11}$, $(CT)_{12}$, $(CT)_{17}$, $(CT)_{18}$, $(CT)_{19}$, $(CT)_{20}$, $(CT)_{21}$ and $(CT)_{22}$. Of the 499 rice accessions, 294 had $(CT)_{11}$ allele and 118 had the $(CT)_{18}$ allele, indicating that these two alleles were the most frequent in this study. Most of the landraces had the $(CT)_{11}$ allele, and some unique alleles were only found in landraces, such as $(CT)_{12}$, $(CT)_{21}$ and $(CT)_{22}$ (Table 2). Most of the breeding lines were associated with $(CT)_{11}$, $(CT)_{17}$ and $(CT)_{18}$ alleles, and $(CT)_{17}$, $(CT)_{19}$ and $(CT)_{20}$ alleles were only detected in the breeding lines. Two breeding lines were polymorphic, one (BP097) contained $(CT)_{11}$ and (and $CT)_{18}$ alleles, and the other (BP470) $(CT)_{18}$ and $(CT)_{20}$. The new alleles detected in this study, such as $(CT)_8$, $(CT)_{10}$, $(CT)_{12}$, $(CT)_{21}$ and $(CT)_{22}$, that were not detected in our previous report (Bao et al. 2002b) were sequenced and the DNA sequencing data confirmed the $(CT)_n$ repeat numbers.

A total of 324 rices had the AGGTATAC sequence (G SNP) at the putative leader intron 5′ splice site of the *Wx* gene, while others (175) had AGTTATAC (T SNP) (Fig. 1). Of the landraces, only four had the T SNP, whereas the other 168 had the G SNP. Among the breeding lines, there was an approximately equal number of G SNP and T SNP genotypes. Two breeding lines contained both G and T SNPs. The wild rice (BP580) had the $(CT)_{10}$ allele and G SNP.

The polymorphism information content (PIC) of SSRs and SNPs in the breeding line set was 0.649 and 0.499, respectively, higher than those in the landrace set (Table 2), indicating that the breeding lines had higher polymorphism at the *Wx* SSR and SNP loci than the landraces.

Microsatellites in the *SS1* gene

A previous study (Bao et al. 2002b) reported that there were three alleles identified in the region of the *SS1* gene, i.e., $(AC)_2$...$TCC(TC)_{11}$...$(TC)_5C(ACC)_{11}$ (*SSS*-A allele), $(AC)_3$...$TCT(TC)_6$...$(TC)_4C(ACC)_9$ (*SSS*-B allele) and $(AC)_3$...$TCT(TC)_6$...$(TC)_4C(ACC)_8$ (*SSS*-C allele). Of all the 499 accessions of nonwaxy rice, 142 had *SSS*-A allele, 145 had *SSS*-B allele, and 176 had *SSS*-C allele. The distribution of these alleles

Table 2 Alleles and PIC of starch-synthesizing gene markers and their distributions in the landrace set (n = 172) and the breeding line set (n = 327)

Markers	Alleles	No. of accessions[a]		
		Landrace	Breeding line	Total
Wx SSR[b]	8	6	2	8
	10	1	1	2
	11	151	143	294
	12	10	0	10
	17	0	54	54
	18	1	117	118
	19	0	4	4
	20	0	3	3
	21	2	1	3
	22	1	0	1
	PIC	0.224	0.649	0.581
Wx SNP	T	4	171	175
	G	168	154	322
	PIC	0.045	0.499	0.456
SS1 SSR[c]	*SSS*-A	100	42	142
	SSS-B	32	113	145
	SSS-C	6	170	176
	SSS -D	34	2	36
	PIC	0.587	0.594	0.705
SBE1 SSR[d]	*SBE*-A	168	252	420
	SBE-B	4	10	14
	SBE-C	0	62	62
	SBE-D	0	1	1
	PIC	0.045	0.398	0.269
SBE1 STS	NI	172	263	435
	I	0	60	60
	PIC	0	0.303	0.213
SBE3 SNP	C	113	244	357
	G	59	81	140
	PIC	0.451	0.374	0.405

PIC polymorphism information content, *NI* no transposon insertion STS, *I* containing transposon insertion STS

[a] The polymorphic rice accessions (with more than one allele present within accession) were not included in the analysis

[b] No. of $(CT)_n$ repeats

[c] *SSS*-A: $(AC)_2$...TCC$(TC)_{11}$...$(TC)_5C(ACC)_{11}$; *SSS*-B: $(AC)_3$...TCT$(TC)_6$...$(TC)_4C(ACC)_9$; *SSS*-C: $(AC)_3$...TCT$(TC)_6$...$(TC)_4C(ACC)_8$; *SSS*-D: $(AC)_2$...TCC$(TC)_{10}$...$(TC)_4C(ACC)_9$

[d] *SBE*-A: CTCTCGGGCGA...$(CT)_{10}$; *SBE*-B: CTCTCGGGCGA...$(CT)_8$; *SBE*-C: $(CT)_8$; *SBE*-D: CTCTCGGGCGA...$(CT)_9$

was different between the landraces and breeding lines; 100 landraces contained *SSS*-A allele, whereas most of the breeding lines contained *SSS*-B (113 accessions) and *SSS*-C (170 accessions) alleles. In addition to these three composite alleles, another allele, $(AC)_2$...TCC$(TC)_{10}$...$(TC)_4C(ACC)_9$ (*SSS*-D allele) was identified in 36 rice accessions, and the sequencing analysis confirmed the repeat numbers within the sequence (Table 1). However, it should be noted that only two rice accessions (BP367 and BP442) with the *SSS*-D allele were identified in the breeding lines, whereas all other 34 accessions with the allele were landraces. The wild rice (BP580) had the *SSS*-C allele (Table 2).

The breeding line set and the landrace set had a similar PIC (0.59) for this marker, but the PIC of all the accessions was 0.705, the highest value among all the markers (Table 2).

Microsatellites and STS in *SBE1* gene

A previous study (Bao et al. 2002b) reported that there were three microsatellite alleles amplified in a region of *SBE* gene, i.e., CTCTCGGGCGA...$(CT)_{10}$ (*SBE*-A allele), CTCTCGGGCGA...$(CT)_8$ (*SBE*-B allele) and the $(CT)_8$ repeats alone without the insertion sequence (*SBE*-C allele). Of all the 499 accessions of nonwaxy rice, 420 had the *SBE*-A allele, 14 had the *SBE*-B allele, and 62 had the *SBE*-C allele. The allele distribution varied between the landraces and the breeding lines. In the landraces, 168 accessions had the *SBE*-A allele, and four had the *SBE*-B allele. However, 252 of 327 breeding lines had the *SBE*-A allele, 10 had the *SBE*-B allele and 62 had the *SBE*-C allele. Two accessions (BP468 and BP469) had both *SBE*-B and *SBE*-C alleles. In addition to the three alleles identified before, one new allele, CTCTCGGGCGA... $(CT)_9$ (*SBE*-D allele) was found in only one rice accession, IR1552 (BP021). This allele was confirmed by DNA sequencing analysis.

There were 60 rice accessions having the transposon insertion STS allele (insertion STS), 435 accessions lacking the insertion (Table 2; Fig. 1), whereas four breeding lines (BP465, BP468, BP469 and BP483) having both of the alleles. In contrast, all the landraces lacked the insertion STS. The wild rice had the *SBE-C* microsatellite allele but no insertion STS.

The PIC of *SBE1* SSR was very low (0.045) and that of *SBE1* STS was zero in the landrace set because all the landraces lacked the insertion STS. The PIC value was 0.398 for *SBE1* SSR and 0.303 for *SBE1* STS in the breeding line set (Table 2).

SNP in the *SBE3* gene

A total of 357 rice accessions had the C SNP allele while 140 accessions had the G SNP allele in the *SBE3* gene, whereas the other two accessions (BP477 and BP542) were polymorphic (Table 2, Fig. 2). In the breeding lines, 244 and 81 had the C SNP and G SNP, respectively. In the landraces, 113 had the C SNP and 59 had the G SNP. The wild rice (BP580) had the G SNP allele.

The PIC of this SNP was 0.405 among all accessions, with a higher value in landrace (0.451) than in breeding

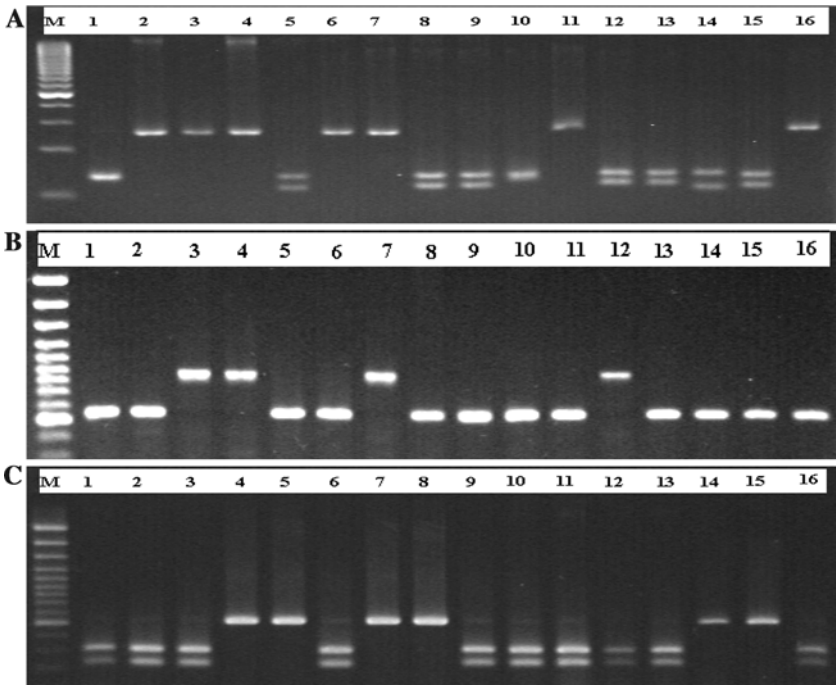

Fig. 1 Polymorphism of the SNP in the *Wx* (**a**), the STS in *SBE1*(**b**) and the SNP in the *SBE3* (**c**) genes. **a** Polymorphism of the SNP in the *Wx* gene detected using restriction endonuclease *Acc*I, lanes from 1 to 16 are accessions BP015 (G SNP), BP017 (T SNP), BP018 (T), BP019 (T), BP020 (G), BP024 (T), BP026 (T), BP027 (G), BP028 (G), BP047 (G), BP054 (T), BP115 (G), BP131 (G), BP201 (G), BP269 (G) and BP274 (T). Variation in banding pattern among the G SNP alleles is caused by $(CT)_n$ SSR polymorphism within the gene. **b** Polymorphism of the STS in *SBE1*, lanes from 1 to 16 are accessions BP003 (no insertion STS, NI), BP015 (NI), BP017 (insertion STS, I), BP018 (I), BP020 (NI), BP021 (NI), BP032 (I), BP033 (NI), BP034 (NI), BP037 (NI), BP038 (NI), BP339 (I), BP545 (NI), BP546 (NI), BP547 (NI) and BP548 (NI). **c** Polymorphism of the SNP in the *SBE3* detected using restriction endonuclease *Spe*I, lanes from 1 to 16 are accessions BP010 (G SNP), BP017 (G), BP018 (G), BP019 (C SNP), BP024 (C), BP032 (G), BP051 (C), BP053 (C), BP056 (G), BP334 (G), BP335 (G), BP384 (G), BP514 (G), BP523 (C), BP531 (C) and BP534 (G)

lines (0.374). It should be noted that only this SNP marker displayed higher polymorphism in landraces than in breeding lines (Table 2).

The differences in starch physicochemical properties among rices with different alleles of SSR, SNP and STS

Table 3 summarized the relationship between different SSR, STS and SNP allele classes and starch physicochemical properties. The apparent amylose content (AAC) of the allele classes of *Wx* $(CT)_8$, $(CT)_{10}$, $(CT)_{11}$ and $(CT)_{12}$ were more than 28%, significantly higher than the AAC of other allele classes with higher numbers of CT repeats, e.g., $(CT)_{17}$, $(CT)_{18}$ and $(CT)_{19}$, whose AAC was less than 23% (Table 3). The other physicochemical traits were also significantly different among different *Wx* $(CT)_n$ classes, but the traits between $(CT)_{11}$ and $(CT)_{12}$, and between $(CT)_{17}$ and $(CT)_{18}$ were similar. In contrast, the pasting temperature (PT) was similar among all *Wx* $(CT)_n$ classes.

Of the four SSR allele classes of *SS1* gene, *SSS*-A and *SSS*-D had similar AAC (~28%), PV (~250 RVU), HPV (~185 RVU), CPV (~334 RVU), BD (~63 RVU), SB (86 RVU), PT (76°C), ADH (~–34) and COH (0.55). These parameters were higher than those of the *SSS*-B and *SSS*-C classes. The *SSS*-B class had smaller BD, ADH and COH but higher AAC, HPV, CPV, SB, PT and HD than the *SSS*-C class.

The three *SBE1* allele classes also showed differences in starch quality parameters. The *SBE*-B and *SBE*-C classes had similar AAC (~20%), which was significantly lower than that of *SBE*-A classes (24.8%). The *SBE*-A class had higher PV, HPV, CPV and HD but lower ADH and COH than the *SBE*-B and *SBE*-C classes.

The different SNP alleles in *Wx* and *SBE3* and STS alleles in *SBE1* gene also differed significantly in almost all starch parameters (Table 3).

The *Wx* SSR and *Wx* SNP alone could explain nearly 90% of the total variation in AAC in all nonwaxy rice (Table 4). These two markers could also

Table 3 Comparison of the mean values of starch physiochemical properties of the SSR, SNP and STS allele classes in 491 nonwaxy rice accessions

Markers	Alleles	No. of Accessions	AAC (%)	PV	HPV	CPV	BD	SB	PT (°C)	HD (g)	ADH (gs)	COH
Wx SSR	8	8	29.4 a	193.6 b	130.3 b	261.3 c	63.3 abc	67.7 abc	75.9 a	43.3 a	–36.4 c	0.53 c
	10	2	28.0 a	140.0 c	96.4 c	190.0 d	43.6 c	50.1 bcd	73.4 a	34.8 a	–27.8 abc	0.55 c
	11	293	28.3 a	248.0 a	188.8 a	341.8 a	59.2 bc	93.9 a	76.1 a	38.2 a	–35.8 c	0.54 c
	12	10	28.9 a	251.6 a	186.7 a	328.5 ab	64.9 abc	76.9 ab	76.0 a	42.7 a	–33.9 c	0.53 c
	17	53	16.4 c	240.2 a	162.9 ab	277.8 c	77.3 ab	37.6 bcd	72.2 a	13.1 c	–19.4 abc	0.69 a
	18	114	15.0 cd	247.3 a	157.2 ab	272.5 c	90.1 a	25.2 cd	73.8 a	12.6 c	–17.7 abc	0.69 a
	19	4	20.9 b	226.7 ab	166.0 ab	290.3 bc	60.7 abc	63.6 a-d	72.0 a	23.5 b	–32.2 bc	0.63 b
	20	3	23.1 b	221.1 ab	143.2 b	278.1 c	77.9 ab	57.0 a-d	74.7 a	24.0 b	–28.2 abc	0.60 b
	21	3	13.2 d	239.8 a	160.9 ab	261.7 c	78.7 ab	22.1 d	72.1 a	11.1 c	–12.7 a	0.73 a
	22	1	12.4 d	232.2 ab	165.5 ab	269.5 c	66.7 abc	37.3 bcd	71.7 a	13.0 c	–14.8 ab	0.71 a
Wx SNP	T	170	15.8 b	245.3 a	159.5 b	274.1 b	85.8 a	28.8 b	73.2 b	12.6 b	–18.1 a	0.69 a
	G	321	28.2 a	245.3 a	185.6 a	336.9 a	59.7 b	91.6 a	76.0 a	38.1 a	–35.6 b	0.54 b
SS1 SSR	A	142	28.3 a	251.4 a	187.0 a	337.1 a	64.3 b	85.8 a	76.2 a	39.3 a	–35.9 b	0.54 c
	B	144	25.9 b	240.6 b	179.1 a	327.1 a	61.5 b	86.5 a	75.6 a	32.9 c	–32.5 b	0.57 b
	C	169	17.2 c	243.7 ab	163.9 b	282.7 b	79.8 a	39.0 b	73.4 b	16.3 d	–20.8 a	0.67 a
	D	36	27.5 a	247.7 ab	184.9 a	333.3 a	62.8 b	85.6 a	76.2 a	36.0 b	–33.6 b	0.55 c
SBE1 SSR	A	416	24.8 a	247.3 a	178.7 a	321.5 a	68.6 a	74.1 a	75.5 a	31.8 a	–31.1 b	0.58 c
	B	14	20.3 b	232.0 b	158.1 b	293.2 b	73.8 a	61.3 a	74.9 a	24.7 b	–26.2 ab	0.62 b
	C	60	16.7 b	236.9 ab	167.7 ab	279.4 b	69.2 a	42.5 b	71.8 b	13.4 c	–19.7 a	0.69 a
SBE1 STS	NI	431	24.7 a	246.5 a	177.8 a	320.2 a	68.6 a	73.7 a	75.5 a	31.5 a	–30.9 a	0.58 b
	I	60	16.7 b	236.9 b	167.7 b	279.4 b	69.2 a	42.5 b	71.7 b	13.4 b	–19.7 b	0.69 a
SBE3 SNP	C	354	24.5 a	246.3 a	178.1 a	321.8 a	68.2 a	75.5 a	75.4 a	30.6 a	–30.6 a	0.58 a
	G	137	21.8 b	242.6 a	172.7 a	298.1 b	69.9 a	55.5 b	74.1 b	25.9 b	–26.9 b	0.62 b

Means having a different letter are significantly different ($P < 0.05$). Eight polymorphic breeding lines (BP097, BP465, BP468, BP469, BP470, BP477, BP483 and BP542) were not included in the analysis

AAC apparent amylose content, *PV* peak viscosity, *HPV* hot paste viscosity, *CPV* cool paste viscosity, *BD* breakdown viscosity, *SB* setback viscosity, *PT* pasting temperature, *HD* gel hardness, *ADH* adhesiveness, *COH* cohesiveness. The values of PV, HPV, CPV, BD and SB are given as Rapid Visco Unit (RVU)

Table 4 Variations of the starch physicochemical properties explained (r^2 value) by each individual marker and by all the markers using the general linear model analysis of 491 accessions of nonwaxy rice

Marker[a]	AAC	PV	HPV	CPV	BD	SB	PT	HD	ADH	COH
Wx SSR	0.903	0.117	0.339	0.560	0.359	0.587	0.234	0.772	0.361	0.828
Wx SNP	0.893	NS	0.195	0.435	0.321	0.558	0.203	0.755	0.354	0.820
SS1 SSR	0.545	NS	0.118	0.277	0.137	0.313	0.175	0.484	0.213	0.526
SBE1 SSR	0.175	0.064	0.053	0.123	NS	0.069	0.178	0.193	0.075	0.223
SBE1 STS	0.162	NS	NS	0.087	NS	0.065	0.170	0.181	0.069	0.211
SBE3 SNP	0.034	NS	NS	0.055	NS	0.050	0.039	NS	NS	0.040
All markers	0.922	0.292	0.462	0.655	0.525	0.676	0.370	0.811	0.409	0.856

The values listed are all significant at $P < 0.0001$; *NS* not significant. Eight polymorphic breeding lines (BP097, BP465, BP468, BP469, BP470, BP477, BP483 and BP542) were not included in the analysis

[a] See Table 3 for the abbreviations

explain most of the variations for all other traits except for PV and PT. Each of the other four markers alone could contribute significantly to the variations in the starch quality traits, but their contributions were much smaller than that of *Wx* SSR or *Wx* SNP. All the six molecular markers together could explain 92.2, 81.1 and 85.6% of the total variations in AAC, HD and COH, respectively. They could also explain more than 40.9% of the variations for HPV, CPV, BD, SB and ADH. However, only 29.2 and 37% of the total variations could be explained for PV and PT, respectively, by all six molecular markers (Table 4).

The difference in starch quality parameters within the same *Wx* SSR classes

It has been shown previously that rice with similar AAC still display differences in eating and cooking quality. Previous studies (Ayres et al. 1997; Bergman et al. 2001; Shu et al. 1999) indicated that though *Wx*

SSR or SNP alone or in combination could differentiate high or low AAC rice accessions, they could not explain all the variations in AAC. The other variations in starch physicochemical properties could be explained, at least in part, by molecular markers other than *Wx* SSR or SNP. There were 293 and 114 rices having the $(CT)_{11}$ and $(CT)_{18}$ allelic background, respectively, and the starch physicochemical properties among the marker allele classes were compared (Table 5).

All 292 $(CT)_{11}$ rices excluding BP577 (an *amylose extender* mutant) had high or very high AAC, ranging from 22.5 to 32.6% (22.5% in one accession, and >24.1% in all others). The CPV, SB and HD were significantly different between the *SBE3* SNP alleles (Table 5). The *SSS*-D allele class had lower AAC (27.3%) than the *SSS*-A, *SSS*–B and *SSS*-C classes (>28%). The *SSS*-B and *SSS*-C classes had smaller PV and BD than the other two allele classes. All 114 $(CT)_{18}$ rice accessions had low or intermediate AAC, ranging from 7.9 to 22.8%. Different *SBE*3 SNP and *SS1* SSR alleles had similar starch quality parameters (Table 5). In contrast, the *SBE1* STS allele classes differed in all the starch quality parameters except for CPV and COH. These results indicated that other molecular markers could be used to explain the variations within the same *Wx* SSR allele classes.

Discussion

More alleles of the microsatellites in starch synthesizing genes were identified in nonwaxy rice in the present study than in any previous studies (e.g., Ayres et al. 1997; Shu et al. 1999; Bergman et al. 2001; Bao et al. 2002b), which may result from more rice samples being included for genotyping in this study, such as wider diversities of landraces, cultivars and breeding lines. The $(CT)_n$ microsatellite in the upstream of the putative 5′- leader intron splice site of rice *Wx* gene has been widely studied. Ayres et al. (1997) and Shu et al. (1999) reported eight $(CT)_n$ microsatellite alleles with n = 8, 11, 14, 16, 17, 18, 19, and 20. Bergman et al. (2001) reported an additional allele, $(CT)_{10}$. Prathepha and Baimai (2004) found only five of the alleles with n = 11, 16, 17, 18, 19 in Thailand rice. In the present study, a total of ten alleles were identified with n = 8, 10, 11, 12, 17, 18, 19, 20, 21 and 22 in the nonwaxy rice samples, most of which being Chinese rice. The alleles, $(CT)_{12}$, $(CT)_{21}$ and $(CT)_{22}$ are reported here for the first time. However, we did not find the $(CT)_{14}$ and $(CT)_{16}$ alleles in our rice materials. The $(CT)_{16}$ allele has been reported in waxy rice (Bao et al. 2002b) and Ayres et al. (1997) have also found one $(CT)_{16}$ waxy rice from China. It should be noted that nonwaxy rice has more $(CT)_n$ alleles than waxy rice; only four alleles, $(CT)_{16}$, $(CT)_{17}$, $(CT)_{18}$ and $(CT)_{19}$, have been consistently identified in waxy rice (Bao et al. 2002b; Han et al. 2004, Prathepha and Baimai 2004). Why different $(CT)_n$ SSR alleles present in the waxy and nonwaxy rice needs further study.

Our previous study identified three alleles in the *SS1* gene in waxy rice (Bao et al. 2002b). These three alleles are also present in nonwaxy rice with one new allele, *SSS*-D, identified in the present study. Three microsatellite alleles in *SBE1* were found in both waxy

Table 5 Comparison of the mean values of starch physiochemical properties of the SSR, SNP and STS allele classes among rice accessions with the same *Wx SSR* allele

Markers	Allele	No. of accession	AAC (%)	PV (RVU)	HPV (RVU)	CPV (RVU)	BD (RVU)	SB (RVU)	PT (°C)	HD (g)	ADH (gs)	COH
Among 292 accessions with *Wx* $(CT)_{11}$												
SS1 SSR	*SSS*-A	128	28.2 a	255.0 a	190.2 a	342.3 a	64.8 a	87.3 b	76.1 ab	39.4 a	–36.5 a	0.54 b
	SSS-B	111	28.5 a	242.3 bc	188.8 a	344.8 a	53.5 b	102.4 a	76.1 ab	37.5 ab	–35.3 a	0.54 ab
	SSS-C	20	28.4 a	237.2 c	187.7 a	340.0 a	50.7b	102.8 a	75.3 b	39.5 a	–38.1 a	0.53 b
	SSS-D	33	27.3 b	251.1 ab	186.5 a	338.1 a	63.4 a	87.0 b	76.4 a	35.2 b	–34.3 a	0.55a
SBE3 SNP	C	237	28.2 a	247.6 a	189.5 a	344.7 a	58.1 b	97.2 a	76.1 a	37.5 b	–35.8 a	0.54 a
	G	55	28.2 a	252.5 a	187.5 a	333.5 b	65.0 a	81.0 b	76.0 a	40.9 a	–36.2 a	0.54 a
Among 114 accessions with *Wx* $(CT)_{18}$												
SS1 SSR[a]	*SSS*-B	16	15.4 a	248.6 a	151.1 a	269.8 a	97.5 a	21.2 a	74.8 a	12.8 a	–18.5 a	0.68 a
	SSS-C	97	14.9 a	247.4 a	158.3 a	272 5 a	89.0 a	25.2 a	73.6 a	12.6 a	–17.5 a	0.69 a
SBE1 STS	NI	92	14.7 b	250.4 a	154.7 b	270.7 a	95.6 a	20.3 b	74.2 a	12.5 b	–17.3 a	0.69 a
	I	22	16.3 a	234.3 b	167.6 a	280.0 a	66.7 b	45.7 a	71.8 b	13.2 a	–19.6 b	0.69 a
SBE3 SNP	C	78	14.6 b	250.2 a	154.9 a	272.3 a	95.3 a	22.1 a	74.1 a	12.6 a	–17.5 a	0.69 a
	G	36	15.7 a	241.0 a	162.2 a	273.0 a	78.8 b	32.0 a	73.1 a	12.8 a	–18.1 a	0.69 a

See Table 3 for the abbreviations. Means having a different letter are significantly different ($P < 0.05$)

[a] Only accession BP451 had *SSS*-A allele, so it was not included in the comparison

rice (Bao et al. 2002b) and nonwaxy rice (the present study). With more rice samples included in the present study, however, one new allele, *SBE*-D, was identified in nonwaxy rice (BP021).

The microsatellite alleles were not equally distributed in the landraces and breeding lines (Table 2). The landraces had ten accessions with $(CT)_{12}$ and one with $(CT)_{22}$, but these alleles were not present in the breeding lines. In contrast, $(CT)_{17}$, $(CT)_{19}$ and $(CT)_{20}$ were only found in the breeding lines. On the other hand, 34 of the 36 rices with the *SSS*-D allele were landraces, but the *SBE*-C allele was not present in the landraces. The difference in the distribution of microsatellite alleles could result from domestication processes or different breeding activities, if the favorable alleles were selected and are still utilized in the current breeding programs. *Wx* SSR, SNP, *SBE1* SSR and STS loci in the breeding lines all had higher PIC values than in the landraces, suggesting that breeding activities may broaden or maintain the genetic diversities. Different starch properties are needed for specific product processing, leading to the divergent breeding aims, and thus different alleles of the starch genes could be selected. Although the PIC of *SS1* microsatellites is similar between the landraces and the breeding lines, the allele frequencies are very different between the two sets of rice samples. The rarer alleles in the landraces appeared to be selected for in the breeding lines, whereas the common alleles in the landraces are under represented in the breeding lines, especially the *SSS*-D allele. This may reflect artificial selection for *SSS*-B and *SSS*-C alleles or against *SSS*-D allele in recent breeding programs, if these alleles are closely associated with the starch physicochemical properties. In contrast, the higher PIC of *SBE3* SNPs in the landraces may suggest this site was not under directional selection.

Different alleles of starch synthesizing genes may function differently to affect the starch physicochemical properties. The analysis of $(CT)_n$ alleles and G/T SNPs of the *Wx* gene in relation to the amylose contents has clearly shown that the alleles with fewer repeats ($n \leq 12$) are highly associated with higher AAC, and those with more repeats are highly associated with lower AAC in the present and previous studies (Ayres et al. 1997; Shu et al. 1999; Bergman et al. 2001). Rices with $(CT)_8$, $(CT)_{10}$, $(CT)_{11}$, or $(CT)_{12}$ had more than 28% of AAC in this study (Table 3). Only one rice with $(CT)_8$ and 21.8% of AAC was identified by Ayres et al. (1997). Recently, Fitzgerald (2004) summarized the observed relationships between the AAC and the number of CT repeats (Bligh et al. 1995; Ayres et al. 1997; Bergman et al. 2001; Bao et al. 2002b) and indicated that different population structure (*indica*, *japonica* and *javanica*) had different *Wx* (CT) alleles. *Wx* microsatellite and SNP also differentiate other starch properties, such as pasting viscosity and gel hardness (Table 3). As QTL mapping has indicated that pasting viscosity and gel texture parameters were controlled by the *Wx* gene (Bao et al. 2000; 2004), it is not surprising that *Wx* microsatellite and SNP also differentiate these traits and explain a large part of their total variations (Table 4). Different *SS1* microsatellite classes have different starch physicochemical properties (Table 3), which can explain 55% of the total variation in AAC, and more than 30% variation in SB, HD, and COH (Table 4). Larkin et al. (2003) also showed that the *SS1* microsatellite had a lesser effect than *Wx* and additive effects on AAC and paste viscosity characteristics, but they suggested that this may primarily be a linkage effect since the locus is only 5–10 cM away from *Wx* locus (Tanaka et al. 1995). Though statistic results show that other SSR, SNP and STS are also significantly related to the starch physicochemical properties (Tables 3, 4), their contributions are relatively small as compared to *Wx* SSR and SNP (Table 4). Larkin et al. (2003) reported that the *SBE1* and *SBE3* alleles themselves were not significant for AAC and paste viscosity characteristics in a rice population segregating for these traits, suggesting that this could be related to the specific cross examined or reflect the expanding role that *Wx* appears to have in the control of starch fine structure, contributing to the different viscosity characteristics.

Another interesting finding in this study is that the other molecular markers may be used to explain the variations among rices within the same allele classes of *Wx* SSR. If all variations in AAC and other starch physicochemical properties can be explained by the *Wx* alleles, all rices with the same allele should have the same AAC and other properties. This is apparently not the case, because rices with the same *Wx* SSR allele may still differ in these traits, resulting in different textural properties of cooked rice. Thus, *Wx* SSR and SNP alleles could sometimes fall short as a predictor of these properties. The other molecular markers can then act as additional predictors and contribute to explain at least part of the variations in the starch properties. For example, we found in this study that the AAC of *SSS*-D class was significantly lower than other allele classes in the 292 rice accessions with the same $(CT)_{11}$ allele of *Wx* SSR and that the *SS1* SSR allele classes can also differentiate PV, BD, SB, PT, HD and COH. The CPV, SB and HD

were also significantly different between the *SBE3* SNP alleles (Table 5). Among the 114 rices with the same $(CT)_{18}$ allele, we found that the *SBE3* SNP allele classes had different AAC but were almost the same in all other traits. However, the *SBE1* STS alleles can differentiate almost all the starch physicochemical properties (Table 5). The QTLs of other starch genes, e.g., *SBE1* and *SBE3*, can be detected in the genetic population derived from parents with similar AAC, e.g., IR64 and Azucena (Bao et al. 2002c). Different alleles of *SS1*, *SBE1* and *SBE3* can differentiate starch pasting viscosity and thermal properties among waxy rices that have <2% AAC (Bao et al. 2002b; Han et al. 2004). Larkin et al. (2003) reported that the *SBE1* and *SBE3* alleles had significant interactions with *Wx* alleles though they had no direct relation with AAC themselves. All these results indicate that there are still differences among rices with the same *Wx* allele background but these variations can be explained in part by other starch synthesizing genes.

Functional molecular markers derived from within or around genes may causally affect phenotypic trait variation and they can be used in breeding programs without prior mapping if the relationships between marker polymorphisms and target traits have been established (Andersen and Lübberstedt 2003). In this study, the relationships between the alleles of starch synthesizing genes and starch physicochemical properties have been found, and the allele polymorphisms can be easily used in rice breeding for improvement of starch properties, and eating, cooking and processing qualities. However, the roles of microsatellite motif, SNP or STS in the function of the genes are still poorly understood, only the functions of G/T SNP and $(CT)_n$ repeats in the *Wx* gene in relation to amylose content have been clearly established (Bligh et al 1998; Cai et al. 1998; Hirano et al. 1998; Isshiki et al. 1998). The microsatellite in the *SS1* gene is located in the untranslated region just before the transcription start site (GenBank accession no. D16202) (Bao et al. 2002b). The $(CT)_n$ microsatellite in the *SBE1* gene is located in the intron 2 (GenBank accession no. D10838) (Bao et al. 2002b). The transposon STS is in the promoter region of the *SBE1* gene (Han et al. 2004). The C/G SNP in the *SBE3* gene is in the 3′-untranslated region (Han et al. 2004). All these molecular markers do not affect any of the amino acid sequences of the target genes. Also, no evidence has shown that polymorphisms at these loci affect the target gene expressions. These markers themselves may not have direct functions, but they can still relate to the variations in phenotypic traits through linkage disequilibrium between the marker site and the functional domain of the target genes.

All the genes examined in this paper encode key enzymes in the synthesis of amylose and amylopectin (Nakamura 2002; Satoh et al. 2003; Tanaka et al. 1995; Tomlinson and Denyer 2003). However, other genes may play additional roles in influencing the physicochemical behavior of rice starch, such as *SSIIa*. Different alleles of *SSIIa* in rice contribute to different fine structure of amylopectin, leading to different gelatinization temperature of starch (Umemoto et al. 2002). It is not well understood whether different alleles of these genes exist in natural populations and whether these alleles are correlated with starch physicochemical properties (Umemoto et al. 2004; Nakamura et al. 2005; Fitzgerald 2004). Therefore, further studies are needed to investigate the roles these genes play in relation to naturally occurring variation in starch properties.

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