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ORIGINAL PAPER

Genetic analysis of starch paste viscosity parameters in glutinous rice (*Oryza sativa* L.)

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Abstract Starch paste viscosity plays an important role in estimating the cooking, eating, and processing quality of rice. The inheritance of starch paste viscosity in glutinous rice remains undefined. In the present study, 118 glutinous rice accessions were collected, and the genotypes of 17 starch synthesis-related genes (SSRG) were analyzed by using 43 gene-specific molecular markers. Association analysis indicated that 10 of 17 SSRGs were involved in controlling the rapid visco analyzer (RVA) profile parameters. Among these, the *PUL* gene was identified to play an important role in control of peak viscosity (PKV), hot paste viscosity (HPV), cool paste viscosity (CPV), breakdown viscosity (BDV), peak time (PeT), and paste temperature (PaT) in glutinous rice. Other SSRGs involved only a few RVA profile parameters. Furthermore, interactions between SSRGs were found being responsible for PeT, PaT, and BDV. Some of the RVA parameters, including PKV, HPV, CPV, CSV, and PaT, were mainly governed by single SSRG, whereas other parameters, such as BDV, SBV, and PeT, were controlled by a few SSRGs, functioning cooperatively. Further, three near-isogenic lines (NIL) of a *japonica* glutinous cv. Suyunuo as genetic background, with *PUL*, *SSIII-1*, and *SSIII-2* alleles replaced with those of *indica* cv. Guichao 2, were employed to verify the genetic effects of the various genes, and the results were consistent with those obtained from the association analysis. These findings indicated that starch paste viscosity in glutinous rice had a complex genetic system, and the *PUL* gene played an important role in determining the RVA profile parameters in glutinous rice. These results provide important information for potentially improving the quality of glutinous rice.

Keywords Glutinous rice · Association analysis · Starch synthesis related genes · RVA profile parameters

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Introduction

Rice is one of the most important crops as it provides the staple food for half of the world's population, and high yield and good quality are two priorities in rice production. Due to the successful utilization of the semi-dwarf gene *sd-1* and heterosis technology, grain yield has been dramatically improved over the past several decades. However, far more improvements in the grain quality are required to meet the demand of consumers in rice producing areas.

Cooking and eating quality is widely considered as a major criteria for the grain quality, mainly determined by three physical and chemical indices, amylose content (AC) (Juliano 1985), gel consistency (GC) (Cagampang et al. 1973), and gelatinization temperature (GT) (Little et al.

1958). In the past decades, many researches focused on the genetic natures of these physicochemical indices to identify the genes controlling grain quality (He et al. 1999; Tan et al. 1999; Bao et al. 2000a, 2002; Lanceras et al. 2000; Septiningsih et al. 2003; Aluko et al. 2004; Tian et al. 2005; Fan et al. 2005; Wang et al. 2007). The accumulated results showed that AC and GC were largely determined by *Wx* gene, locating on chromosome 6 and encoding the granule-bound starch synthase (He et al. 1999; Tan et al. 1999; Septiningsih et al. 2003; Fan et al. 2005; Wang et al. 2007; Mikami et al. 2008). Similarly, GT was mainly controlled by *ALK* (or *SSII-3*) gene (He et al. 1999), encoding soluble starch synthase IIa (Umemoto et al. 2002; Umemoto and Aoki 2005; Gao et al. 2003).

Association analysis is a powerful tool for studying genetic loci involved in the inheritance of complex traits (Abdurakhmonov and Abdukarimov 2008; Yu and Buckler 2006; Remington et al. 2001), and it has been successfully exploited in plant molecular genetics (Whitt et al. 2002; Wilson et al. 2004; Aranzana et al. 2005; Cockram et al. 2008). Through association analysis approach, a complex network controlling eating and cooking quality was identified, revealing that different characteristics were controlled by different gene combinations, and the genetic diversity of SSRGs greatly accounted for the varied grain quality (Tian et al. 2009).

Starch is composed of two forms, amylose and amylopectin. The fact that the cooking and eating quality varied among cultivars with similar AC suggests that the structure of amylopectin also has an effect in determining the physical and chemical properties (Juliano 1985). Moreover, the true genetic effects of other starch synthesis-related genes (SSRGs) are usually masked by the *Wx* gene (Bao et al. 2002), and the interactions between *Wx* and other SSRGs were often detected (He et al. 2006; Wu et al. 2006). The exact genetic effects of other SSRGs in shaping the rice grain quality are unclear yet.

Glutinous rice lacks starch amylose, which constitutes up to 30% of the total starch in non-glutinous rice endosperm. Glutinous rice is generally reserved for use in festival foods and desserts, although it also serves as the staple food in upland regions of Southeast Asia (Roder et al. 1996). Currently, it is widely used as an industry resource due to its specific property of lacking amylose in endosperm. Similar to common *indica*/*japonica* cultivars, the glutinous rice varieties vary dramatically with respect to the eating and cooking quality, but the mechanism underlying the eating and cooking quality in glutinous rice is still unclear. Most researches on rice grain quality have been conducted on non-glutinous rice (Bao and Xia 1999; Bao et al. 2000a, b; Liu et al. 2004; Ge et al. 2005; He et al. 2006; Wang et al. 2007), only a few being on glutinous rice (Han et al. 2004; Bao et al. 2006). Therefore, we urgently need to reveal the genetic basis of eating and cooking quality in glutinous rice, and to provide the foundation for quality improvement in this food product.

It is well known that there are multiple isoforms of four classes of enzymes involved in starch synthesis, including ADP-glucose pyrophosphorylase (AGPase), starch synthase (SS), starch branching enzymes (SBE) and starch debranching enzymes (DBE), and each enzyme functions distinctly (Myers et al. 2000; Nakamura 2002). In the absence of the *Wx* gene, the glutinous rice has little or no amylose and is mainly composed of amylopectin, enabling us to study the genetic effects of SSRGs in glutinous rice. In previous studies, the effects of only a few genes were investigated in glutinous rice (Bao et al. 2006; Han et al. 2004). However, ~20 genes encoding four classes of enzymes are involved in amylopectin biosynthesis, and each gene plays a distinct role (Nakamura 2002). Therefore, until now, the genetic basis of glutinous rice quality remains obscure. Such questions as which gene(s) is the key factor(s) in determining the glutinous rice quality, and how they function remain to be addressed. The answers to these questions will be helpful in elucidating the mechanism of glutinous rice grain cooking and eating quality.

Recently, the RVA profile of starch paste viscosity was employed to evaluate rice eating and cooking quality (Juliano 1996). The RVA profile has become increasingly popular for investigating the viscosity property, since it requires only a small sample size and the procedure is easy to perform (Bao and Xia 1999). Here, we used a population of 118 glutinous rice varieties to investigate the genetic effects of 17 SSRGs involved in the starch biosynthesis system on formation of the grain RVA profile through association analysis. Additionally, the genes controlling grain RVA profile parameters without the effect of *Wx* were identified and verified with near-isogenic SSRG lines.

Materials and methods

Plant materials

In the present study, we collected 118 glutinous rice accessions from China and International Rice Research Institute (Table 1). Most of the accessions are *indica* and *japonica* types in *O. sativa*, while eight accessions belong to *O. glaberrima*. These glutinous rice accessions were planted in the experimental farm of Yangzhou University and Hainan in 2006 and 2007 in the rice growing season. At maturity, the seeds from each accession were harvested for RVA profile measurement.

Table 1 Glutinous rice varieties employed in the present study

Code	Accession	Description	Origin	Code	Accession	Description	Origin
1	Hongmangxiangjingnuo	*Japonica*	China	60	Yangnuo No. 2	*Japonica*	China
2	Xiangjingnuo	*Japonica*	China	61	Jingnuo No. 6	*Indica*	China
3	Xiangjingnuo-1	*Japonica*	China	62	Lixiaonuo	*Indica*	China
4	Xiangmangnuo	*Japonica*	China	63	Baotanuo	*Indica*	China
5	Xiangzhunuo	*Japonica*	China	64	Xiangjingnuo 103	*Japonica*	China
6	Xiangzhunuoxuan	*Japonica*	China	65	Yangnuo No. 5	*Japonica*	China
7	Wujinxiangnuo	*Japonica*	China	66	Xiannuo 201	*Indica*	China
8	Jinggunuo	*Japonica*	China	67	Yangzhou No. 4	*Japonica*	China
9	Hongkenuo	*Japonica*	China	68	Gehuxiangnuo	*Japonica*	China
10	Hongkenuo-1	*Japonica*	China	69	Jiangzhouxiangnuo	*Japonica*	China
11	Baikenuo	*Japonica*	China	70	Yangxiannuo 32-2	*Indica*	China
12	Baikenuo-1	*Japonica*	China	71	Yandao No. 5	*Indica*	China
13	Baikenuo-2	*Japonica*	China	72	Xiangjingnuo 259	*Japonica*	China
14	Hongmangnuo	*Japonica*	China	73	Huinuo	*Japonica*	China
15	Hongmangnuo-1	*Japonica*	China	74	99-25	*Japonica*	China
16	Hongmangnuo-2	*Japonica*	China	75	Shuangfeng No. 4	*Japonica*	China
17	Jintannuo	*Japonica*	China	76	Yaxuenuo	*Indica*	China
18	Jintannuo-1	*Japonica*	China	77	Baixiangnuo	*Japonica*	China
19	Liyangnuo	*Japonica*	China	78	Shangnongxiangnuo	*Japonica*	China
20	Liyangnuo-1	*Japonica*	China	79	Taihunuo	*Japonica*	China
21	Wannuodao	*Japonica*	China	80	Shiwuyenuo	*Japonica*	China
22	Wannuodao-1	*Japonica*	China	81	Jingnuo 96103	*Japonica*	China
23	Shuangjiangqinnuodao	*Japonica*	China	82	Guanglingxiangnuo	*Japonica*	China
24	Xueliqinnuodao	*Japonica*	China	83	T002	*Japonica*	China
25	Xicainuo	*Japonica*	China	84	Changsiruanzhan	*Indica*	China
26	Changjingnuo	*Japonica*	China	85	Hongzaonuo	*Indica*	China
27	Shuijinnuo	*Japonica*	China	86	G3-66	*Japonica*	China
28	Huangjingnuo	*Japonica*	China	87	Cungunuo	*Indica*	China
29	Putaonuo	*Japonica*	China	88	TAPOL	*Indica*	Philippines
30	Putaonuo-1	*Japonica*	China	89	BLACK GORA S.N. 109	*Indica*	India
31	Zhongqiuputaonuo	*Japonica*	China	90	CHALBYEO	*Japonica*	Korea
32	Huajiaonuo	*Japonica*	China	91	SHIKOKU MOCHI	*Japonica*	Japan
33	Huakenuo	*Japonica*	China	92	SHAN KIU JU	*Japonica*	China
34	Bainuodao	*Japonica*	China	93	KININPOL	*Japonica*	Philippines
35	Zhuzhongnuo	*Japonica*	China	94	MINAMI-HATA MOCHI	*Japonica*	Japan
36	Yangnuodao	*Japonica*	China	95	ARC 10283	*Javanica*	India
37	Yangnuodao-1	*Japonica*	China	96	CHIBA-A-HO-MOCHI	*Javanica*	Brazil
38	Zinuo	*Japonica*	China	97	HSINCHU GLUTTNOUS	*Javanica*	Brazil
39	Hongmangnuo-3	*Japonica*	China	98	PULUT PUTEH	*Javanica*	Malaysia
40	Hongmangnuo-4	*Japonica*	China	99	DAENG MAFAI	Intermediate	Thailand
41	Jintainuo	*Japonica*	China	100	ANANDI	Intermediate	Nepal
42	Huangnuo	*Japonica*	China	101	MIMIDAM	Intermediate	Bangladesh
43	Hongkenuo-2	*Japonica*	China	102	TININTA(MALAGKIT)	Intermediate	Philippines
44	Baikenuo-3	*Japonica*	China	103	AIBAIKOU	Intermediate	China
45	Jiangyinnuo	*Japonica*	China	104	KATUPA-AI	Intermediate	Taiwan
46	Zaonuodao	*Japonica*	China	105	KETAN TRENGGALEK	Intermediate	Indonesia
47	Chushunuo	*Japonica*	China	106	ARC 13163	Intermediate	India
48	Shixingnuodao	*Japonica*	China	107	HAWM OM	*O. glaberima*	Thailand

Table 1 continued

Code	Accession	Description	Origin	Code	Accession	Description	Origin
49	Zhendao No2	*Japonica*	China	108	SUWEON 311	*O. glaberima*	Korea
50	Yannuo 98-9	*Japonica*	China	109	TAICHUNG SEN GLUTINOUS 1	*O. glaberima*	Taiwan
51	Xuenuo	*Indica*	China	110	TAICHUNG SEN GLUTINOUS 2	*O. glaberima*	Taiwan
52	Xinxiangnuo	*Japonica*	China	111	HIRAKAWA OKUTE	*O. glaberima*	Japan
53	57697	*Indica*	China	112	PULUTAN(PUTI)	*O. glaberima*	Philippines
54	535	*Indica*	China	113	XIANG N004	*O. glaberima*	China
55	Xiaomakenuo	*Indica*	China	114	HUANG SI NOO	*O. glaberima*	China
56	Bendixiangnuo	*Indica*	China	115	Makenuo	*Japonica*	China
57	Baiainuo	*Indica*	China	116	Zhenzhunuo	*Japonica*	China
58	3401	*Indica*	China	117	Heijienuo	*Japonica*	China
59	Henuo	*Indica*	China	118	Liutiaonuo	*Japonica*	China

No. 88–114 were introduced from Genetic Resources Center of IRRI

In order to verify the genetic effects of the identified genes obtained from association analysis approach, the near-isogenic lines (NIL) of the corresponding genes were developed. In the process of development of NIL, a typical *indica* cultivar Guichao 2 (inferior quality) and a glutinous *japonica* cultivar Suyunuo (good quality), were selected as donor and recipient, respectively, to produce a cross, and then continuously backcrossed for eight generations by molecular marker-aided selection. Finally, a series of NILs were obtained, and the investigation of their genetic background was conducted with 80 SSR markers covering the entire genome and 17 markers specific to starch synthesis genes. These NILs were also planted in the experimental farm of Yangzhou University in the rice growing season in 2008, and the seeds were harvested for RVA profile assessment.

RVA profile measurement

The RVA profiles were measured on a rapid visco analyser (RVA) (Model No. RVA-3D, Newport Scientific, Sydney, Australia), according to the Standard Method AACC61-02 given by the American Association of Cereal Chemists (2000). Briefly, approximately 3 g rice flour was mixed with 25 ml water; a paddle was placed in the canister and rotated at 960 rpm for 10 s to disperse the rice sample. The viscosity was evaluated using a constant paddle rotation of 160 rpm. The sequential temperature curve for a 12.5 min test was as follows: (1) incubate at 50°C for 1.0 min; (2) increase to 95°C; (3) keep at 95°C for 1.4 min; (4) cool down to 50°C; and (5) hold at 50°C for 1.4 min. Viscosity values were recorded in centipose (cp). Starch viscosity characteristics included the following original components: peak viscosity (PKV), hot paste viscosity (HPV), and cool paste viscosity (CPV). Three secondary parameters including breakdown (BDV), setback (SBV), and consistency (CSV) were calculated based on the original data: BDV = PKV − HPV, SBV = CPV − PKV and CSV = CPV − HPV. In addition, pasting temperature (temperature of the initial viscosity increase, PaT) and pasting time (time of the initial viscosity increase, PeT) were also recorded.

Molecular marker development and assessment

In order to determine the diversity of SSRGs at the genomic level, a total of 13 representative cultivars, including 6 *japonica* cv. Nipponbare, Chunjiang 06, Wuyujing 7, Suyunuo, Taihunuo, Jiangzhouxiangnuo, and 7 *indica* cv. 93-11, 9308, Minghui 63, Longtefu, Guichao 2, TN 1 and Zhenshan 97, were selected and 17 SSRGs (including *AGPiso*, *AGPsma*, *AGPlar*, *GBSS II*, *SSI, SSII-1*, *SSII-2*, *SSII-3*, *SSIII-1*, *SSIII-2*, *SSIV-1*, *SSIV-2*, *SBE1*, *SBE3*, *SBE4*, *ISA*, and *PUL*) from each cultivars were then cloned and sequenced (Tian et al. 2009). Based on the genomic diversity of SSRGs, 43 STS/CAPs markers (Table 2) were developed and subsequently employed for genotyping 118 glutinous accessions (Tian et al. 2010).

Population structure and association analysis

The population structure was evaluated by using the STRUCTURE program (Pritchard et al. 2000) with 45 simple sequence repeats (SSR) distributed on 12 chromosomes in rice, including RM259, RM5, RM128, RM14, RM211, RM475, RM263, RM525, RM16, RM251, RM489, RM520, RM514, RM335, RM471, RM252, RM255, RM122, RM289, RM587, RM412, RM242, RM205, RM528, RM585, RM11, RM180, RM234, RM336, RM264, RM308, RM223, RM316, RM566, OSR28, RM333, RM216, RM258, RM474, RM286, RM332, RM441, RM17, RM101, and

Table 2 Gene-tagged markers for SSRGs

Genes name	Markers name	Primer sequence	Markers type
AGPlar	AGPlar M1	[F] CGTTCAGGTTCAGGCAATCA	STS
		[R] GGAAGGGTGGTGATGTGGAG	
	AGPlar M2	[F] GCGTGAACTGAACATCCATCT	CAPS (*Tsp*45I[a])
		[R] GGTTCAAGCCTTCAGGTCAG	
AGPiso	AGPiso M2	[F] CAATCGCTGCCATCGGTTG	STS
		[R] TTCCACATCGTTAGGTACACG	
	AGPiso M3	[F] TGGAATGGGAACTCTATTATTGG	CAPS (*Eco*RI)
		[R] TCCCAACCTCTACCTTCAAATG	
AGPsma	AGPsma M1	[F] TCTATTCTCAGCCCTCCAACC	STS
		[R] GTGTGTTTAGAGGTGCTTTTCG	
	AGPsma M2	[F] TACGCTATGCTCTTGAAAC	STS
		[R] TATCTTCCCAGTAACCATCA	
GBSSII	GBSSII M1	[F] TTGCTGCGAATTATCTGCG	STS
		[R] ACCTCCTCCCACTTCTTTGC	
SSI	SSI M2	[F] CTTCTATCCATTCCTTAATCCCA	STS
		[R] ATGCTATTGATGTTAAGAGGGC	
	SSI M3	[F] GACCCACCTCGCTATCTGTTG	CAPS (*Apa*I)
		[R] GGAAACACCAGACATCAACCAG	
SSII-1	SSII-1 M1	[F] CACCCCACCGTTCTACTATGC	STS
		[R] TCCATAGTTTCATTGAGATTGCTC	
	SSII-1 M2	[F] CAAGTTGGTGACGATAGTGATGA	CAPS (*Age*I)
		[R] AACAGAGCCTCCATTACCTTTAC	
	SSII-1 M3	[F] AGAGATCAAATCGTGGAAC	STS
		[R] TGGAGTGAAGTAGTGGAAT	
	SSII-1 M4	[F] ATCTTTAGACGATTAGCG	STS
		[R] AAGTCACAAGTAGAAGGG	
SSII-2	SSII-2 M1	[F] AGATTTGAACTCAGGACTTGGTG	STS
		[R] TCTATGGGCTCTATCCTTACTAGG	
	SSII-2 M3	[F]ACAGTATGTTTGCCTCAGCG	STS
		[R] GTAAATCCACCCAGCCAGTC	
SSII-3	SSII-3 M1	[F] CCAATACCGTAAACTAGCGACTATG	STS
		[R] TACAGGTAGAATGGCAGTGGTG	
	SSII-3 M2	[F] GGTTCTCGGTGAAGATGGC	CAPS (*Ban*II)
		[R] GTGGTCCCAGCTGAGGTCC	
	SSII-3 M3	[F] AACTGACTCATACACGGATAACG	CAPS (*Nhe*I)
		[R] CACGCACGAACGGAAACC	
SSIII-1	SSIII-1 M1	[F] AAGAAGGGAAGGGAGTCAGC	SSR
		[R] GCCATCTCCATTGCCAGC	
	SSIII-1 M2	[F] CAAGCAATGATTCAGGCACA	CAPS (*Eco*RI)
		[R] GGAGACAGGAGCAAAAGGC	
	SSIII-1 M3	[F] CAAATCAACTGTAAGTGCTGGAG	CAPS (*Nde*I)
		[R] GAGAACGGAGAAAATGGCAT	
SSIII-2	SSIII-2 M1	[F] AAGTCCTTCGGCTTACTATTCC	CAPS (*Xba*I)
		[R] GGAGAAGGAACATAACAGGGAC	
	SSIII-2 M2	[F] GAACTTGTGCCTTAAGCTGACTG	STS
		[R] GGAATAGTAAGCCGAAGGACTT	
SSIV-1	SSIV-1 M1	[F] CATTGTGTCTTGAAGTCTGTGCT	CAPS (*Nde*I)
		[R] CGATGGGTTAGTGCTGTGG	

Table 2 continued

Genes name	Markers name	Primer sequence	Markers type
SSIV-2	SSIV-2 M1	[F] CTTCTGATTGATGGTTGGTTGC	CAPS (*Sph*I)
		[R] GGAAGAATAATCTCTACTAGGTGGC	
	SSIV-2 M2	[F] TTCCCTTGGTGGTGCGTG	STS
		[R] TAAAGCGTTCCGACAGTA	
	SSIV-2 M3	[F] TCAAGTATGGTTTACCTATG	CAPS (*Eco*72I)
		[R] TTTCCCAATGACTTCTAA	
SBE1	SBE1 M1	[F] TGCTACATAACACGCATACAAAGT	STS
		[R] AGACAAAAGCGAAAGGTAATGAG	
	SBE1 M2	[F] GTGGGGAAAACAAGTAAGTCTG	STS
		[R] AGTTCCATCAGAAGAATCAGGG	
	SBE1 M3	[F] GGAAATGGGAGTCGCC	STS
		[R] CGAAGAAACCACGCTCA	
	SBE1 M4	[F] ATTGTTGCTGAAGATGTTT	CAPS (*Taq*I)
		[R] ACGGTTGATGGTAGGTG	
SBE3	SBE3 M2	[F] GTGGGGTTCTCAACTTAGC	STS
		[R] CATCAGCATTGTTAGGCAG	
SBE4	SBE4 M1	[F] CACCAATTATATTAGCGTGCTCC	STS
		[R] CGTGGCTCTTGGCTCTCTTG	
	SBE4 M2	[F] CCATCACCTCAAATACATCACTC	STS
		[R] AGACTGGAATGCCCCTTAGG	
ISA	ISA M1	[F] ATAGATGCTAATGTGATGTGGC	STS
		[R] TGGTATAGGCACAACCGTAGA	
	ISA M2	[F] ACAAGCACACGACACCTA	CAPS (*Hin*dIII)
		[R] CAACAAACCAAACTCATT	
	ISA M3	[F] TGTGGGAATACCTTCAACTG	STS
		[R] ATAAAACCCTTACAGGCTTG	
PUL	PUL M1	[F] AGAGAAGGAGAAAGAAGTGGAGAC	STS
		[R] GTCCAAACTGAATCACTCAATCG	
	PUL M2	[F] CCACCATTAAAGCATCATCAAC	STS
		[R] AGTTGTTATATTTTAGGATGGATGG	
	PUL M3	[F] CTGTATGGACTGAGTAGTCGATGG	STS
		[R] TGAGCCTCATCTGCCAGAGT	
	PUL M4	[F]TACACCATCCTCACTACCA	STS
		[R] GCAACATCTAAAACACCAA	
	PUL M5	[F]ATTGGCATTTGTAAGTTTC	STS
		[R] CAATCTTGGTTTTATCCTG	
	PUL M6	[F]ATTTAACTGTATGGACTGAG	STS
		[R] GATACCAACCAAACAAGA	

[a] Represents the enzymes used for CAPS

RM519. The number of subgroups was determined to be two based on an admixture and linkage model, which agreed with prior population information. The resulting Q-values were obtained from the STRUCTURE program. The relative kinship (K) matrix was calculated using the software package SPAGeDi (Hardy and Vekemans 2002). Association analysis followed the unified mixed model previously reported (Yu et al. 2005), using SAS 9.0.

Statistical analysis

A *t* test program in SPSS 10.0 was employed to determine the presence of significant differences between the RVA

profile parameters of Suyunuo and those of NIL-*PUL*, NIL-*SSIII-1,* and NIL-*SSIII-2*.

Results

Phenotypic variations within the glutinous rice accessions

We planted 118 glutinous rice accessions in Yangzhou and Hainan in 2006 and 2007, respectively, and seeds from each accession were harvested for assessments of amylose content and RVA parameters. Due to its expected significant effect on RVA parameters, the amylose contents for 118 glutinous accessions were first assessed. The results showed that the amylose contents of all samples were less than 3% (data not shown), indicating that the 118 accessions in the present study were truly glutinous rice. The RVA measurements were then performed. It was found that the RVA data collected over the 2 years were very similar, and significant correlations were observed (data not shown). Therefore, we averaged the 2-year RVA data for the following analysis. The accessions were ordered according to PKV value, and eight RVA parameters of the 118 accessions are shown in Fig. 1 and Table 3.

As shown in Fig. 1, most of the RVA parameters, including PKV, HPV, CPV, BDV, and CSV, of *japonica* accessions were generally less than those of the *indica* accessions. For example, there were 77 accessions whose PKV values were <1,700 cp; of which, 69 accessions belonged to the *japonica* type, whereas, only seven accessions belonged to the intermediate type, and one was *O. glaberima*. In the 41 accessions whose PKV were bigger than 1,700 cp, there were 20 *indica*, 10 *japonica*, 3 *javanica* and 3 intermediate types. As for SBV, the reverse trend was observed. This result suggested that the genes in control of the RVA profile parameters have differentiated, at least between the *indica*/*japonica* subspecies. Moreover,

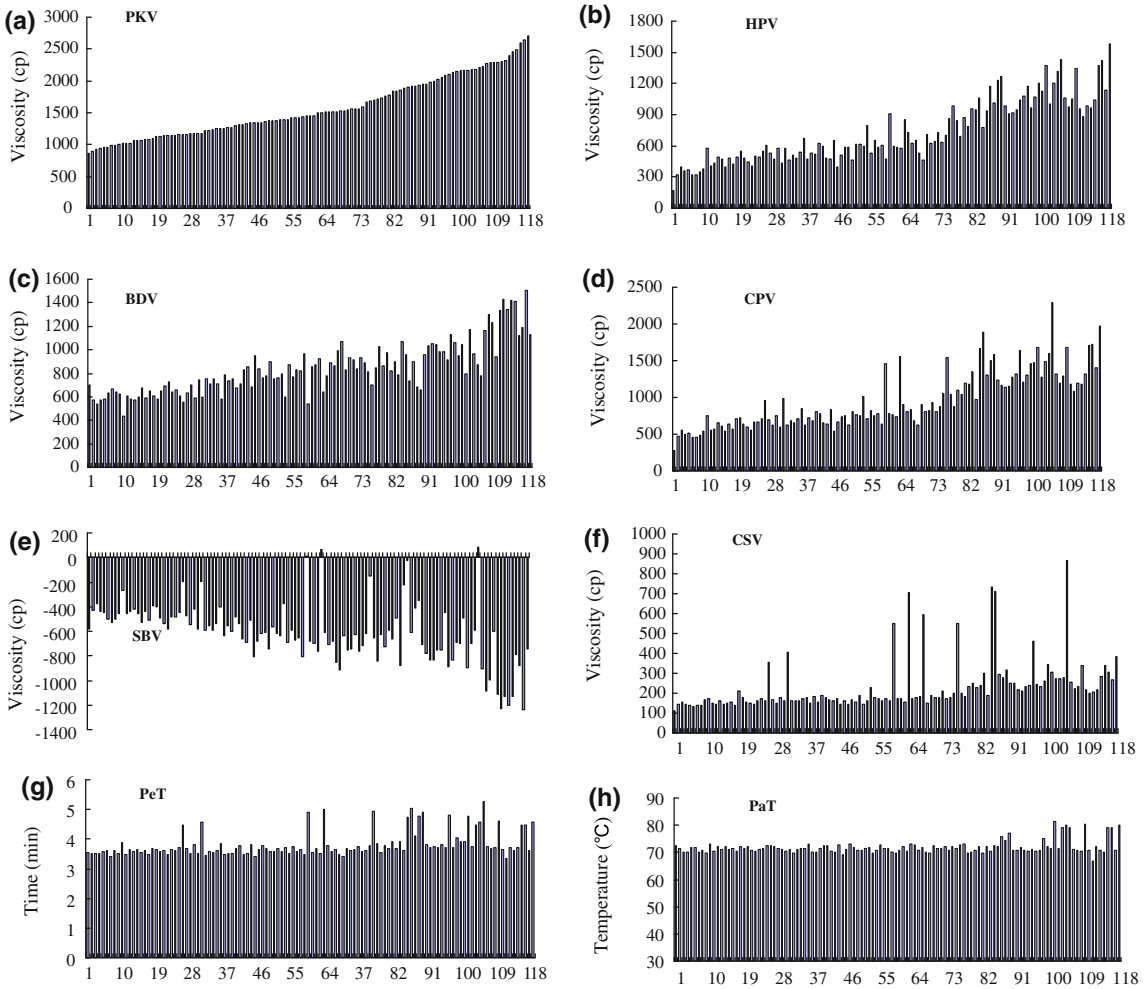

Fig. 1 RVA profile parameters of 118 glutinous rice accessions. **a–h** PKV, HPV, BDV, CPV, SBV, CSV, PeT, and PaT, respectively. The *x*-axis denotes the accession code, which is ordered based on the PKV value

Table 3 Statistical analysis of RVA profile parameters in 118 glutinous rice accessions

Parameters	Mean	Standard deviation	Minimum	Maximum
PKV (cp)	1,570	517.4	610	3,084
HPV (cp)	731.7	401.34	139	1,875
BDV (cp)	838.6	281.1	285	1,712
CPV (cp)	958.5	531.4	242	3,363
SBV (cp)	−611.8	347.9	−1,516	952
CSV (cp)	230.4	186.6	98	1,488
PeT (min)	3.8	0.6	3	6.6
PaT (°C)	71.9	2.9	64.1	82.35

The results were obtained by averaging 2 years' data in 2006 and 2007

most of the RVA parameters, such as PKV, HPV, CPV, SBV, BDV, and CSV, varied widely among the 118 glutinous accessions (Table 3). For example, the PKV value ranged from 868 to 2,711 cp, with an average value of 1,570 cp. By contrast, PaT and PeT had relatively small variations, with PaT in most of accessions being very close to about 70°C, few exceptions at 80°C. As for PeT, most of the accessions were ~3.6 min, while only 18 accessions (15%) were ~5 min (Table 3). These results suggest that, in the present study, the 118 glutinous accessions are representative in terms of rice grain quality and are qualified for genetic analysis of RVA parameters.

It also should be noted that several accessions have dramatically higher or lower values in CPV, SBV, CSV, and PeT, distinct from the other accessions, including No. 26 (Putaonuo, *japonica*), No. 31 (Wujinxiangnuo, *japonica*), No. 59 (Ketan Trenggalek, intermediate), No. 63 (Bainuodao, *japonica*), No. 76 (Hongmangnuo, *japonica*), No. 86 (Suweon 311, *O. glaberima*), and No. 105 (Tininta, intermediate) (Fig. 1d–g). The existence of distinct glutinous rice accessions implies that in glutinous rice germplasm, the genes underlying the eating and cooking quality have differentiated dramatically, although these genes are unknown yet. These distinct accessions may be helpful in the development of special varieties for industry.

Correlation among RVA parameters

To explore the relationship among eight RVA profile parameters, the pairwise correlation analysis was conducted, and the correlation coefficients among eight RVA profile parameters were summarized in Table 4. Interestingly, the significant correlations were found between almost any two parameters; and only three pairwise correlations between BDV and HPV, CPV, CSV did not reach the significant level (Table 4). The result suggested that the eight RVA profile parameters in the 118 glutinous accessions were interdependent, similar to the results by Wang et al. (2007).

Although significant correlations among the eight RVA parameters were observed, many exceptions also existed. For example, in general, PKV was positively correlated with HPV; however, the accession Liutiaonuo had high PKV (2,446 cp), but its HPV was relatively low (734 cp). In addition, CPV in most of the glutinous accessions were <1,500 cp, while some accessions, such as Bainuodao, Liyangnuo, Suweon 311 and Yangxiannuo 32-2, exhibited

Table 4 Correlation analysis among eight RVA profile parameters

	PKV	HPV	BDV	CPV	SBV	CSV	PeT
HPV	0.84217						
	<0.0001						
BDV	0.63821	0.12237					
	<0.0001	**0.0605**					
CPV	0.78015	0.96536	0.05766				
	<0.0001	<0.0001	**0.3779**				
SBV	−0.2953	0.22216	−0.86081	0.36727			
	<0.0001	0.0006	<0.0001	<0.0001			
CSV	0.41498	0.60785	−0.10404	0.77712	0.56982		
	<0.0001	<0.0001	**0.1109**	<0.0001	<0.0001		
PeT	0.41478	0.74557	−0.30104	0.8559	0.69043	0.83403	
	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	
PaT	0.43673	0.68504	−0.17421	0.65111	0.34504	0.38751	0.66857
	<0.0001	<0.0001	0.0073	<0.0001	<0.0001	<0.0001	<0.0001

The number in the upper line is the correlation coefficient, and the number in the lower line indicates the corresponding probability

$P > 0.05$ showed in bold

bigger CPV, more than 1,500 cp. These results primarily suggested that different genetic mechanisms are responsible for the different RVA profile parameters.

Association analysis

In association analysis, the population structure usually plays an important role in identifying target gene loci. Therefore, in order to eliminate the influence of population structure effects on association analysis, we analyzed the population structure by using the STRUCTURE software based on the genotypes of 45 SSR markers covering the entire rice genome. The results showed that 118 glutinous accessions could be divided into two groups (Fig. 2), which fit well with their species-specific properties (*indica* vs. *japonica*). The *Q* value of each accession generated from population structure analysis was used in the following association analysis.

The association analysis result was summarized in Table 5. As shown, three original parameters, PKV, HPV, and CPV were interestingly found to be mainly controlled by the *PUL* gene, suggesting that starch (amylopectin) viscosity was mainly affected by this gene in glutinous rice. By contrast, two secondary parameters, BDV and SBV, several genes were identified to be responsible for their variations. For BDV, four genes, including *AGPlar*, *PUL*, *SSI*, and *SSIV-1*, were shown to be responsible for its variation in 118 glutinous accessions. Seven genes, including *AGPlar1*, *SBE1*, *SBE3*, *ISA*, *SSII-1*, *SSII-2*, and *SSIV-1*, were found to contribute to the SBV variation in the glutinous rice population, of which, *SSIV-1* had the largest effect according to the *F* value. However, for another secondary parameter, CSV, only one gene, *SBE1*, was found to be responsible for its variation.

As for PeT parameter, which reflects the time of the initial viscosity increase, three genes, *SSII-3*, *PUL,* and *AGPlar*, were identified. Among these, *SSII-3* had the largest genetic effect with an additive effect of 0.82°C, apparently functioning as a key factor in determining the PeT parameter. However, only the *PUL* gene was found to associate with PaT variation, which represents the temperature of the initial increase in starch viscosity.

To comprehensively understand the genetic mechanisms underlying the RVA profile parameters, the interactions among 17 genes were analyzed (Fig. 3). The result showed that complex genetic interactions existed for controlling the BDV parameter; the network contained not only four identified genes (*PUL*, *SSI*, *AGPlar,* and *SSIV-1*) when a single gene was considered in association analysis, but also three additional genes, including *ISA, SBE1,* and *SSII-2*, which were not detected to be independently responsible for BDV variations. The fact that the genetic network involved four classes of enzymes, AGPase, SBE, DBE, and SSS, indicated that there was a very complex genetic system underlying BDV performance. Similarly, interactions between *SSII-3* and *PUL* as well as between *SSII-3* and *SSIII-2* were also identified to contribute PaT parameter. Furthermore, an interaction between *PUL* and *SSIII-2* was also found to affect the PeT parameter. Apart from

Table 5 Results of association analysis between SSRG and RVA profile parameters

Trait	SSRG	Estimate	*F* value	*P* value
PKV	*PUL*	442.02	38.07	1.03E−06
HPV	*PUL*	295.09	38.69	3.14E−06
BDV	*AGPlar*	264.25	5.58	1.10E−03
	PUL	138.06	8.33	6.92E−03
	SSI	223.78	14.59	2.54E−04
	SSIV-1	238.97	7.41	6.69E−03
CPV	*PUL*	266.68	26.31	3.93E−04
SBV	*AGPlar*	385.48	9.00	1.53E−04
	SBE1	281.45	8.56	4.47E−03
	SBE3	258.70	7.51	7.45E−03
	ISA	288.56	7.49	7.07E−03
	SSII-1	213.50	6.60	1.07E−02
	SSII-2	397.35	11.12	1.08E−03
	SSIV-1	405.97	13.64	3.17E−04
CSV	*SBE1*	104.41	6.98	8.72E−04
PeT	*AGPlar*	0.51	4.89	7.14E−03
	PUL	0.29	8.75	4.45E−03
	SSII-3	0.82	4.81	1.69E−03
PaT	*PUL*	1.83	12.34	2.34E−03

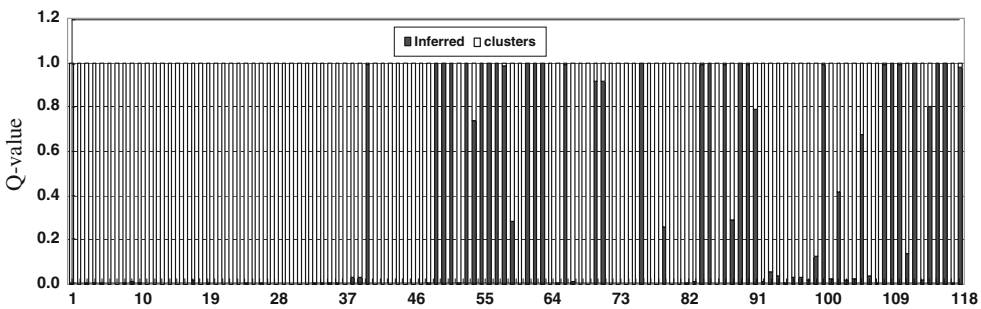

Fig. 2 Population structure analysis. The results of structure analysis indicated that two groups ($K = 2$) exist in this population, and the *Q* value of each individual are shown

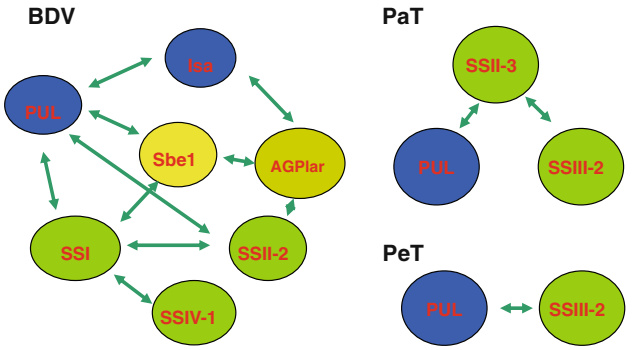

Fig. 3 A diagram showing the genetic interactions controlling BDV, PaT, and PeT parameters. The *arrows* between any two genes indicate the existence of an interaction between them

BDV, PaT, and PeT, we failed to detect any interactions between the other five RVA profile parameters.

Taken together, our analysis revealed that in 17 SSRGs, the *PUL* gene was shown to be heavily involved in the regulation of most of the RVA profile parameters in glutinous rice; the complex genetic systems associated with RVA profile parameters were controlled through both individual SSRGs as well as through interactions between SSRGs.

Verification of genetic effects of SSRG through NILs

To validate the results of the association analysis, three near-isogenic lines, NIL-*PUL*, NIL-*SSIII-1,* and NIL-*SSIII-2*, were employed to evaluate the variations of RVA profile parameters. These three NILs were generated by marker-assisted selection toward target genes *PUL, SSIII-1,* and *SSIII-2* in the progeny of backcrossed population derived from a cross of Suyunuo (a *japonica* glutinous landrace, recipient) × Guichao 2 (an *indica* cultivar, donor). The result was shown in Table 6. When compared to the recurrent parent Suyunuo, significant changes occurred in PKV, HPV, BDV, CPV, and SBV of NIL-*PUL*, whereas CSV, PeT and PaT did not alter significantly. This result was basically consistent with the results obtained in the association analysis, confirming that the *PUL* gene plays an important role in shaping the eating and cooking quality in glutinous rice. However, no any significant changes in RVA parameters were observed in NIL-*SSIII-1* when compared to its recurrent parent Suyunuo. In fact, in the association analysis, *SSIII-1* showed no effects on eight RVA parameters, suggesting that there was no functional differentiation at the *SSIII-1* locus, at least between Guichao 2 and Suyunuo, or that it may be compensated by other SSS. As for NIL-*SSIII-2*, the significant changes in PeT and PaT were detected compared to the recurrent parent Suyunuo, and other RVA profile parameters were

Table 6 Comparison of RVA profile parameters between recurrent parent and NILs

Materials	Sample size	PKV (cp)	HPV (cp)	BDV (cp)	CPV (cp)	CSV (cp)	SBV (cp)	PeT (min)	PaT (°C)
Suyunuo	4	1,037.25 ± 119.76	346.50 ± 52.74	690.75 ± 73.95	491.5 ± 66.38	145 ± 14.63	−545.75 ± 59.97	3.50 ± 0.03	71.05 ± 0.44
NIL-SSIII-1	10	1,030.70 ± 169.74	358.70 ± 59.26	672.00 ± 112.04	513.00 ± 68.38	154.3 ± 12.09	−517.70 ± 109.21	3.47 ± 0.09	70.71 ± 0.81
NIL-PUL	4	1,278 ± 53.63*	470.50 ± 30.69**	807.50 ± 29.15*	625.25 ± 39.48*	154.75 ± 0.49	−652.75 ± 25.32*	3.53 ± 0.05	70.7 ± 0.34
NIL-SSIII-2	4	1,184.75 ± 116.13	364.75 ± 27.50	820.00 ± 97.36	513.50 ± 33.97	148.75 ± 10.72	−617.25 ± 87.18	3.36 ± 0.04**	69.35 ± 0.46**

* Significant at 5% level, **significant at 1% level

not altered significantly; meanwhile, the *SSIII-2* was found to be incorporated into the SSRG interactions for regulating PaT and PeT in association analysis. Therefore, the finding based on three NILs was basically consistent with those from association analysis with respect to genetic effects of SSRGs.

Discussion

Glutinous rice is generally reserved for use in festival foods and desserts, and it also serves as the staple food in the upland regions of Southeast Asia (Roder et al. 1996). Now, the glutinous rice also is widely used as an industry resource. However, little efforts have been made to reveal the genetic mechanism of the starch paste viscosity in the glutinous rice. In the present study, the genetic mechanism of starch viscosity in glutinous rice was analyzed by using the association analysis method, and the results provided an overview of the genetic basis of the starch viscosity profile in glutinous rice. Such finding will potentially benefit future efforts to improve the quality of glutinous rice, and perhaps even of the common *indica* and *japonica* rice.

The paste viscosity profile is considered as a very important predictor of the eating, cooking, and processing quality characteristics of common *indica*/*japonica* rice (Juliano 1985; Shu et al. 1998), Thus, information on the genes affecting paste viscosity parameters will facilitate our efforts to improve rice grain quality, although the relationship between paste viscosity profile and eating and cooking quality in glutinous rice remains unclear yet. In previous studies, Bao et al. (2006) developed several microsatellite markers for the *SSI* and *SBE1* loci on the basis of sequence diversity, and the relationship between the genotypes on *SBE1* and *SSI* loci and starch thermal and retrogradation properties, pasting properties, and swelling volume in 56 glutinous rice accessions were analyzed. The results showed that accessions with different genotypes on the two loci exhibited different starch properties, suggesting that alleles on *SBE1* and *SSI* loci have functionally differentiated. Meanwhile, Han et al. (2004) analyzed the effects of the *SBE1* and *SBE3* genes on the paste viscosity in 40 glutinous rice lines, and the result indicated that polymorphisms on both *SBE1* and *SBE3* loci accounted for ~70% of the observed variations in both hot and cool viscosities, and for ~40% of the observed variations in peak viscosity and consistency. However, in the two aforementioned studies, the population sizes were relatively small, and a few of SSRGs were considered. In particular, the effect of population structure on the gene effects analysis was neglected, and these shortcomings may have led to inaccurate conclusions about the gene functions. In the present study, we employed a larger population (118 glutinous accessions), and most of the SSRGs (17) and population structures were considered as a whole to analyze the genetic behavior of starch paste viscosity parameters; for these reasons, our conclusions may be more reasonable.

In the past decades, more researches on rice grain quality were performed in non-glutinous rice, in which the role of amylopectin in eating and cooking quality is, at least in part, masked by amylose (Bao et al. 2002), whereas in glutinous rice which contains only amylopectin, the eating and cooking quality would be predominantly affected by the properties of this polysaccharide. Hence, some genes with minor effects may possibly be overlooked in non-glutinous rice. Gravois and Webb (1997) previously analyzed the genetic behavior of rice viscosity, and their results showed that PKV, HPV, and CPV were controlled by one major gene (*Wx*). Bao et al. (2000a) mapped the QTL for RVA parameters using a doubled haploid population derived from a cross between an *indica* variety Zai-Ye-Qing 8 and a *japonica* variety Jing-Xi 17, and a total of 20 QTL for six parameters of the RVA profiles were identified at least in one location; meanwhile, only the *Wx* gene was detected significantly in both environments for five traits (HPV, CPV, BDV, CSV, and SBV), indicating that the RVA profiles were mainly governed by the *Wx* locus, although some minor QTL were also identified to be responsible for the RVA profiles (Bao et al. 2000b). These results were further confirmed by Wang et al. (2007), in which a recombinant inbred population derived from a cross between Zhenshan 97 and Delong 208 was used to analyze the genetic basis of RVA profiles. Thus, it is clear that the *Wx* gene is a key determinant in the control of RVA profile parameters in common *indica* and *japonica*. However, in glutinous rice, loss-of-function mutation occurring in the *Wx* gene resulted in the failure of amylose synthesis (Wanchana et al. 2003). Although the *Wx* gene itself does not function in glutinous rice, RVA profile parameters vary widely in glutinous rice accessions. Therefore, it seems that the genetic information gained from analysis of rice grain quality in non-glutinous rice cannot be directly used for glutinous rice analysis and improvement. In the present experiment, the association analysis results showed that most of the SSRGs (10/17) were responsible for the paste viscosity profiles, and the *PUL* gene was shown to play an important role in the control of most of the RVA profile parameters, except for CSV and SBV, in glutinous rice. Unfortunately, the genetic effect of the *PUL* gene was not detected in studies by both Wang et al. (2007) and Bao et al. (2000b). This result may be caused by two factors. First, the genetic populations in both of these studies were derived from a common *indica*/*japonica* cross, in which *Wx* gene works normally and thus, amylose is produced in the rice endosperm. Current

knowledge indicates that the *Wx* gene functions as a major factor in determining RVA profile parameters, and the effect of *PUL* gene can be possibly masked by *Wx*. In fact, when the *PUL* allele in Guichao 2 was substituted with that of Suyunuo, no RVA profile parameters altered significantly when compared to those of Guichao 2 (Wu et al. 2006). Second, the discrepancy between our study and the two reports mentioned above could be that in the Zhai-Ye-Qing 8/Jing-Xi 17 and Zhenshan 97/Delong 208 derived populations, there was no functional differentiation on the *PUL* locus between the two parents, thus leading to the failure of identification of its genetic effect. Taken together, the data suggest that in order to improve grain quality of common *indica* and *japonica* cultivars, we should focus on the selection of favorable alleles on the *Wx* locus in order to regulate the amylose content; however, some amylopectin synthesis related genes, such as *SSII-3*, *PUL*, and *SSIII-2*, which have influence on determining the fine structure of amylopectin, should not be neglected.

It is generally accepted that ADPase, SSS, SBE, and DBE were involved in starch biosynthesis in plants (Nakamura 2002). There were two types of DBE, isoamylase (ISA) and pullulanase (PUL, also known as limit dextrinase or R-enzyme), classified on the basis of their sequence similarity and substrate specificity. Kubo et al. (1999) reported that both ISA and PUL were involved in amylopectin biosynthesis in rice endosperm; they also presumed that ISA played a predominant role in amylopectin synthesis, but PUL was also essential or compensated for the role of ISA in the formation of the amylopectin multiple-cluster structure. The analysis of three PUL-deficient mutants indicated that the short chain (DP $\leq$ 13) of amylopectin in *PUL* mutants was increased, and the average chain length of B2-3 chains was $\sim$3 residues longer compared with that of the wild-type (Fujita et al. 2009). Therefore, it is obvious that *PUL* plays a role in determining the fine structure of amylopectin. In our present study, *PUL* gene was found to be a principal determinant for the variation in RVA profile parameters in glutinous rice, consistent with the results obtained in the analysis of *PUL* mutants.

Both association analysis and NIL analysis indicated that *SSIII-2* was one of regulators of the PaT and PeT parameters, suggesting that this gene indeed plays a role in formation of the fine structure of amylopectin. A previous study showed that the loss-of-function of *SSIII-2* resulted in the reduction of the amylopectin B2–B4 chains with degree of polymerization (DP) $\geq$30 by $\sim$60% of the wild-type values, strongly suggesting *SSIII-2* functions in the elongation of amylopectin B2 to B4 chains (Fujita et al. 2007). Hence, it can be speculated that the chains with DP $\geq$ 30 in amylopectin contributes to the eating and cooking quality, which can be reflected in the PeT and PaT parameters.

Based on the results of our study, it therefore can be deduced that functional differentiations occurred in most of the SSRGs, especially at the *PUL*, *SSII-3,* and *SSIII-2* loci, and different alleles at the *PUL* and *SSIII-2* loci have different genetic effects on RVA profile parameters. In fact, the exploitation of multiple alleles have been widely conducted at the *Wx* and *SSII-3* loci, which were shown as major factors in the control of AC and GT, respectively (Umemoto et al. 2002; He et al. 2006; Wang et al. 2007). To date, at least five alleles at the *Wx* locus were identified in Asian rice germplasm (Mikami et al. 2008), which provides the foundation to explain the continuous variation of AC in Asian rice landraces. Similarly, there are two alleles at the *SSII-3* locus identified in Asian cultivars, and the diversity of the *SSII-3* locus results in the different amylopectin structure and starch quality between *japonica* and *indica* varieties (Umemoto et al. 2002; Nakamura et al. 2005; Bao et al. 2006; Tian et al. 2009), being reflected in the difference of the GT among these varieties. Moreover, in the present study, Guichao 2 and Suyunuo belong to typical *indica* and *japonica* types, respectively, and great sequence diversities were identified on each SSRGs (Tian et al. 2009). The functional differentiations that occurred in a few SSRGs were deduced on the basis of the performances of their NILs. Therefore, with more detailed studies performed, increasing information on allelic diversification of SSRGs loci would be obtained, enabling us to purposefully select a favorable allele on each locus by molecular marker-assisted selection and consequently to develop new varieties to meet the demand of rice consumers.

Although most of the RVA profile parameters were found to be controlled by a few SSRGs, two important parameters, BDV and SBV, previously shown to be closely related to eating and cooking quality (Bao and Xia 1999), were found here to be governed by at least four SSRGs, and various interactions were also found to be in the control of BDV, PaT, and PeT. This result implies that, manipulation of a few SSRGs cannot achieve the goal of improvement of rice grain quality. Moreover, the interactions between SSRGs remain unclear, and more NILs and transgenic lines for SSRGs are needed for evaluating the gene functions and understanding the complex genetic network responsible for starch quality.

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