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The three important traits for cooking and eating quality of rice grains are controlled by a single locus in an elite rice hybrid, Shanyou 63

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Abstract The cooking and eating quality of the rice grain is one of the most serious problems in many rice-producing areas of the world. In this study, we conducted a molecular marker-based genetic analysis of three traits, amylose content (AC), gel consistency (GC) and gelatinization temperature (GT), that are the most important constituents of the cooking and eating quality of rice grains. The materials used in the analysis included F_2 seeds, an $F_{2:3}$ population, and an F_9 recombinant inbred-line population from a cross between the parents of 'Shanyou 63', the most widely grown hybrid in rice production in China. Segregation analyses of these three generations showed that each of the three traits was controlled by a single Mendelian locus. Molecular marker-based QTL (quantitative trait locus) analyses, both by one-way analysis of variance using single marker genotypes and by whole-genome scanning with MAPMAKER/QTL, revealed a single locus that controls the expression of all three traits. This locus coincided with the *Wx* region on the short arm of chromosome 6, indicating that all three traits were either controlled by the *Wx* locus or by a genomic region tightly linked to this locus. This finding has provided clues to resolving the molecular bases of GC and GT in future studies. The results also have direct implications for the quality improvement of rice varieties.

Key words Rice quality · Amylose content · Gel consistency · Gelatinization temperature · Genetic analysis · Molecular marker

Introduction

The grain quality of rice is a complex character composed of many components such as nutritional quality, appearance quality, cooking quality and eating quality, to mention only a few. Each one of these components also consists of many attributes whose values are determined not only by their physical-chemical properties but also by the history and cultural traditions of the people in the human communities who consume the rice.

Grain quality currently represents a major problem in rice production in China and many other rice producing areas of the world. Much of this problem stems from the poor cooking and eating quality of many widely grown varieties, especially the indica varieties. Knowledge accumulated in the past decades indicates that the poor cooking and eating quality is directly related to three attributes of the physical and chemical characteristics of the starch in the endosperm; namely, amylose content (AC) (Webb 1980; Juliano 1985; Unnevehr et al. 1992), gel consistency (GC) (Cagampang et al. 1973) and gelatinization temperature (GT) (Little et al. 1958).

A number of studies have been conducted to characterize the genetic basis of the AC of the endosperm. Results from several studies indicated that AC was specified by a single major locus with modifications of some minor genes (Bollich and Webb 1973; McKenzie and Rutger 1983; Kumar and Khush 1988). However, other studies suggested that AC had a complex genetic basis due to the triploid nature of the endosperm genotype plus the additional complexity of cytoplasmic effects and epistasis (Mo 1993; Pooni et al. 1993).

It has been known for some time that the granule-bound starch synthase (GBSS, EC 2.4.1.11), encoded by the *Waxy* (*Wx*) gene, plays a very important role in determining the AC in grains (Shure et al. 1983; MacDonald and Preiss 1985). In rice two functional alleles of the *Wx* gene have been found to exist that correspond to the AC levels of indica and japonica rice varieties, respectively (Sano et al. 1986). A molecular analysis (Wang et al. 1995) revealed that the splicing pattern of the first intron of the *Wx* gene is highly correlated with the AC level of the grains. Transcripts with the intron completely spliced out would produce high AC, ones with the intron completely unspilced would produce grains with no amylose

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(glutinous rice), while those with partial splicing would produce an intermediate AC.

However, questions still remain as to whether *Wx* is the only locus specifying the AC of the endosperm, or whether additional genetic factors are involved in determining it. Moreover, there has been no reported genetic analysis of the other two physical-chemical properties, GC and GT, that are also closely related to the cooking and eating quality of rice grains.

In the study reported in this paper, making use of high-density molecular-marker linkage maps, we performed a genome-wide analysis of the genetic controls for all three attributes, referred to hereafter as traits, of the cooking and eating quality of rice grains. The populations used for the study were developed from a cross between the parents of 'Shanyou 63', the most widely grown rice hybrid in China. The results thus obtained will also be directly applicable to hybrid rice improvement.

Materials and methods

Genetic materials

The genetic materials were populations of three generations derived from the cross 'Zhenshan 97' × 'Minghui 63' including, (1) a plant of the F_1 hybrid, (2) a population of 241 F_2 plants, and (3) a population of 240 F_9 recombinant inbred lines (RILs) derived from the 241 F_2 plants by single-seed descend. For obtaining measurements of the quality traits, 340 seeds were harvested from the F_1 plant grown in 1996 that were referred to as the F_2 generation, and each of the seeds was individually measured for AC. The seeds harvested from each of the F_2 plants grown in 1996 were bulked for measuring the quality traits and the scores thus obtained for each plant were used in the analysis as representing the F_2 plant. This data set was referred to as the $F_{2:3}$ population, since the seeds were actually of the F_3 generation. The seeds of the RILs were harvested from the F_9 planted in 1997. All the field experiments were planted in the normal rice growing seasons at the Experimental Farm of Huazhong Agricultural University, Wuhan, China. All the seeds were stored at room temperature for a period of at least 3 months after harvesting, before the quality analysis.

Measuring the quality traits

AC

Forty grams of rice grains were de-hulled and milled in duplicates using a miller (manufactured by the Jiading Food and Oil Machinery Factory, Shanghai, China) according to the National Standards NY 147–88 (NY stands for the abbreviation of 'Agricultural' in Chinese spelling). The milled rice from the two duplicates was combined and ground into powder with a Udy Cyclone Sample Mill (Udy Corporation, Colorado, USA), and was then sieved through a 100-mesh sieve. Exactly 25 mg rice flour was gelatinized overnight in 2 ml of 1.0 N NaOH in a water bath set at 50°C. The solution was boiled in the water bath for 10 min and then cooled to room temperature. The cooled solution was extracted three times with 5 ml of butanl:petroleum ether (1:3) to remove the lipid, after which 1.5 ml of 0.4 N KI was added to the solution and mixed. The AC was determined in duplicates with an ART-3 Automatic Titrator according to the manufacturer's instruction (Hirama Laboratories, Japan) in which 1.57 mM KIO_3 was titrated at a speed of 2.5 µl per s to the starch solution. The titration terminal was automatically detected with a sensitivity setting of 3, and the used volume of KIO_3 was transformed into amylose content. Standard amylose solutions (5%, 10%, 15%, 20%, 25% and 30%) were prepared as checks by dissolving pure amylose (Sigma product Cat. No. A-0512) and amylopectin (Sigma product Cat. No. A-8512) in distilled water.

AC measurment for single grains was conducted by starting at the step of placing the brown rice into 2 ml of 1.0 N NaOH at 50°C for gelatinization. The remaining steps including boiling, cooling, and ether-extraction and titration, which were the same as described in the previous paragraph.

GC

The GC was measured in duplicates according to the method of Cagampang et al. (1973). Briefly, 100 mg rice flour was weighed in a 10 mm × 110 mm culture tube, to which 0.2 ml of 95% ethanol containing 0.025% thyml blue was added to prevent clumping of the powder during gelatinization. One milliliter of 0.2 N KOH was added and vortexed thoroughly. The tubes were covered with glass marbles and boiled vigorously in a water bath for 8 min. After standing at room temperature for 5 min, the tubes were put on ice for 20 min, and then laid down horizontally on a table surface. The gel length was measured 1 h later as the distance from the bottom of the tube to the front of the gel migration. The gel length thus obtained provides a measurement of the gel consistency: the longer the distance, the softer the gel.

GT

The GT was measured on the basis of individual grains expressed as the alkali spread value (ASV) using the method of Little et al. (1958) with minor modifications. Each sample was tested three times. Each time, 15 intact milled grains from each F_2 plant ($F_{2:3}$) or RIL were put in a weighing boat, to which 15 ml of 1.7% KOH was added. The grains were carefully separated from each other using a forceps and incubated at 300 C for 23 h to allow spreading of the grains. The spreading value of the grains was scored by visual assessment, essentially as described by Jennings et al. (1979). Grains swollen to the extent of a cottony center and a cloudy collar were given an ASV score 4 and used as a check for scoring the rest of the samples in the population. Grains that were unaffected were given an ASV score of 1, and grains that were dispersed and disappeared completely were given a score of 10. A low ASV corresponds to a high GT, conversely, a high ASV indicates a low GT.

All the measurements of AC and GC for the $F_{2:3}$ plants and the RILs were averaged over replications for further analysis.

Molecular markers and assays

The molecular marker data for the $F_{2:3}$ population were essentially the same as those described in Yu et al. (1997), which consisted of 150 polymorphic loci including restriction fragment length polymorphism (RFLP) and simple sequence repeat (SSR) markers covering all 12 chromosomes. This data set was used for mapping the genes for quality traits of the $F_{2:3}$ data.

We also used three pairs of primers for assaying SSR polymorphisms within the *Wx* gene region. The first pair of primers was designed according to Bligh et al. (1995), with $(CT)_n$ as the targeted SSR sequence. The other two pairs of primers were those of MX4 and MX21 from Xiong et al. (1997). The tagged motif by MX4, $(CT)_n$, was the same as the first pair but the locations of the primer sequences were slightly different. The targeted SSR sequence of MX21 was $(AATT)_n$ which occurred 182 bp downstream from the (CT) repeat. The PCR reaction was carried out essentially as described in Wu and Tanksley (1993) except that the reaction volume was reduced to 10 µl.

One-way analysis of variance of the quality traits with the marker genotypes as groups was conducted using the statistical

package Statistica™ (StatSoft 1995). The whole genome was scanned for quantitative trait loci (QTLs) using MAPMAKER/QTL 1.0 (Lincoln et al. 1992) with a LOD threshold of 2.4.

Results

Inheritance of AC, GC and GT

The endosperm genotypes

Denoting one of the alleles of a locus as *A* and the other allele as *a*, the genotypes of the endosperms of the F_2 population (seeds harvested from an F_1 plant) would segregate in proportions of 1 (*AAA*):3 (*AAa*):3 (*Aaa*):1 (*aaa*). The genotypic compositions of endosperms of the seed bulks of the F_2 plants ($F_{2:3}$) were complicated such that 1/4 of the population would be *AAA*, 1/4 would be *aaa*, and individuals in the remaining 1/2 would each be a mixture of 1 (*AAA*):3 (*AAa*):3 (*Aaa*):1 (*aaa*). Thus the segregation ratio of the population could be either 1:2:1 or 3:1 depending on the genetic effects of the alleles. By contrast, in the RIL population, assuming homozygosity of the locus in all the RILs, the endosperm genotypes would be either *AAA* or *aaa*, thus producing a 1:1 segregation.

AC

The distributions of AC in the three populations are shown in Fig. 1. Transgressive segregation was observed in all three cases. Continuous distributions were observed in both the F_2 seeds (harvested from the F_1) and the $F_{2:3}$ population. These distributions did not allow for a 7:1 ratio in the F_2 seeds. However, in the distribution of the $F_{2:3}$ population, there appeared to be a valley occurring around 18% AC, with individuals above and below this point fitting a 3:1 segregation ratio (Table 1). The lines in the RIL population were clearly separated into two groups, which fits the expected 1:1 ratio (Table 1).

Thus, AC segregation in this cross was clearly controlled by a single Mendelian locus with the allele producing a high AC showing a dominant effect. In addition, the continuous distributions of AC in the F_2 seeds and also the $F_{2:3}$ population (Fig. 1) seemed to indicate a dosage effect of the dominant allele such that genotypes with different numbers of dominant alleles produced different levels of AC.

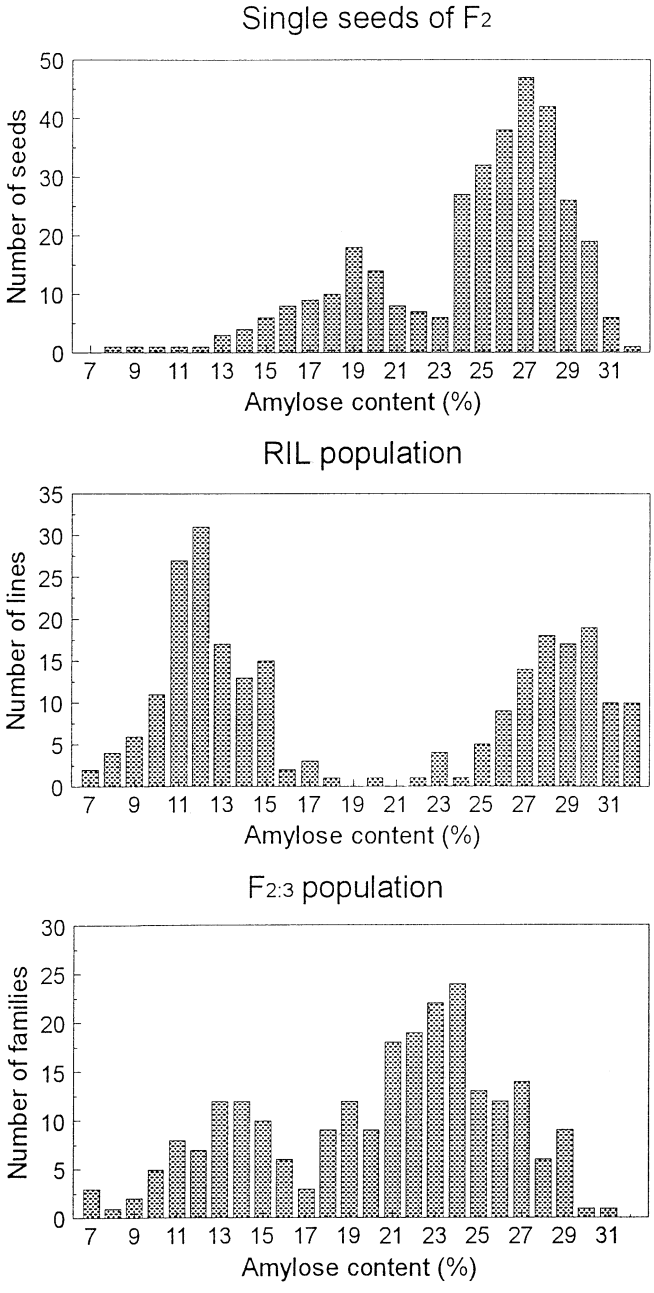

Fig. 1 Distribution of amylose content in different generations of the cross 'Zhenshan 97' × 'Minghui 63'. The AC values of 'Minghui 63' and 'Zhenshan 97' are 15.8% and 27.2%, respectively

GC

Both the F_2 and the RIL populations showed discontinuous distributions, each with a major peak at the low-GC (hard gel) region and a long and flat tail extending from the intermediate-to the high-GC (soft gel) region (Fig. 2). Dividing the $F_{2:3}$ population at the discontinuous point resulted in a hard-gel group and a soft-gel group, which fits a 3:1 segregation ratio (Table 1). Similarly, dividing the RIL population at the discontinuous point resulted in two groups that fit a 1:1 segregation ratio (Table 1). Thus, segregation of GC is also controlled by a single locus in this population.

GT

ASV could clearly separate the individual grains in this population into two distinct parental types: grains that

Table 1 Chi-square tests for single-locus segregation of amylose content (AC), gel consistency (GC) and gelatinization temperature (GT) expressed by the alkali spread value (ASV) in the $F_{2:3}$ and recombinant inbred line (RIL) populations from the 'Zhenshan 97' × 'Minghui 63' cross

Trait	Population	Number of plants (lines)	Number of plants (lines)	Expected ratio	χ^2	P
AC	$F_{2:3}$	176 (>18%)	62 (≤18%)	3:1	0.20	0.653
	RIL	108 (>20.5%)	133 (≤20.5%)	1:1	2.39	0.122
GC	$F_{2:3}$	168 (≤36 mm)	70 (>36 mm)	3:1	2.24	0.134
	RIL	108 (≤35.5 mm)	133 (>35.5 mm)	1:1	2.39	0.122
ASV	$F_{2:3}$	188 (<4)	50 (≥4)	3:1	1.82	0.178
	RIL	135 (<4)	106 (≥4)	1:1	3.25	0.071

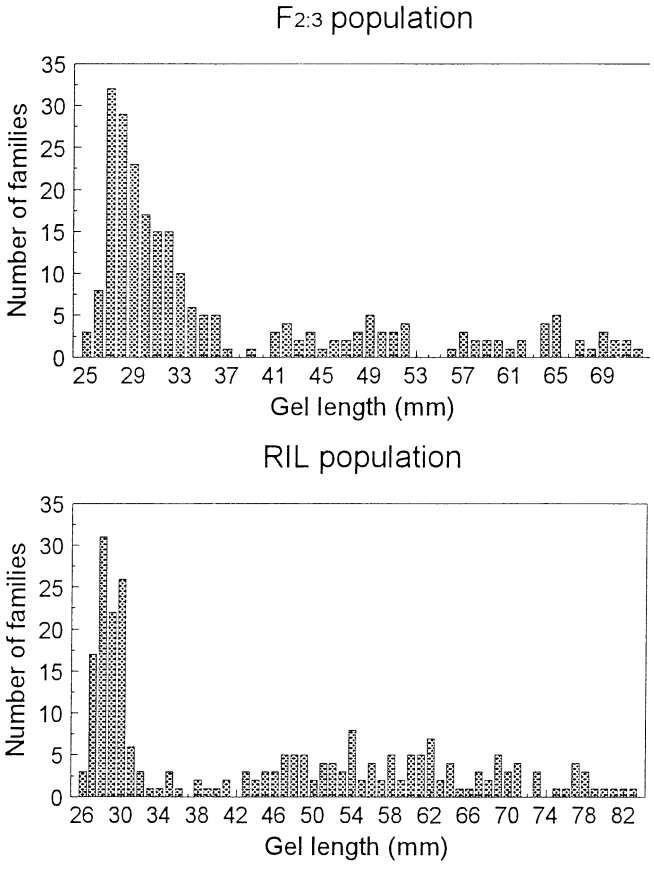

Fig. 2 Distribution of gel consistency (measured by gel length) in the $F_{2:3}$ and RIL populations of the cross 'Zhenshan 97' × 'Minghui 63'. The lengths of 'Minghui 63' and 'Zhenshan 97' are 52.5 mm and 29.0 mm, respectively

did not show obvious spreading (high GT, 'Minghui 63' type) in alkaline solution and grains that showed extensive spreading (low GT, 'Zhenshan 97' type), with very few intermediates. A 3:1 segregation was observed in the $F_{2:3}$ population between the two types of plants, individuals producing seeds that were homozygous for a high GT or a mixture of both types, and individuals producing seeds homozygous for a low GT (Table 1). Similarly, lines of high GT and low GT in the RIL population segregated in a 1:1 ratio (Table 1). Thus, GT segregation in this population is also controlled by a single locus.

The relationship of the three traits

Inspection of the data clearly indicated that the three traits are closely related. An example of such a close association is given in Table 2 using the data of the RIL population for demonstration. With the exception of only four lines, there were only two parental combinations of these three traits: either low AC/high GC (soft gel)/low ASV (high GT) or high AC/low GC (hard gel)/high ASV (low GT). Similar, though less-dramatic, associations were also observed among the three traits in the data of the $F_{2:3}$ population (data not shown).

QTL analysis

To determine the genomic region for the gene(s) controlling these traits, one-way analyses of variance based on the AC and GC data of the $F_{2:3}$ population were conducted using the genotypes of markers representing the entire genome (Yu et al. 1997). Data for GT were not used in this analysis because the variation was not continuous in the populations.

A significant effect was detected only at the genomic region near the distal end of the short arm of chromosome 6 where the *Wx* gene is located (Table 3). Particular attention was also given in the analyses to two other regions. One region was the distal end of the long arm of chromosome 6 where the starch branching enzyme I (Q-enzyme I) is located (Harrington et al. 1997). The other region was on the short arm near the centromere of chromosome 2 (Harushima et al. 1998) where the starch branching enzyme III (Q-enzyme III) is located (Nakamura and Yamanouchi 1992; Nakamura et al. 1994). However, no significant effect was detected in either of these regions.

We scanned the whole genome with MAPMAKER/QTL for QTLs controlling AC and GC, based on the map constructed using the $F_{2:3}$ marker data (Yu et al. 1997). The analysis detected only one peak at exactly the same position for both traits, with a LOD score of 69 for AC and a LOD score of 57 for GC, respectively. This position corresponded very well to the *Wx* locus on chromosome 6 (Fig. 3).

Table 2 The distribution of the three traits AC, GC and ASV in the RIL population using a three-way cross classification

Trait	AC=20.5%		AC>20.5%	
	GC<35.5 mm	GC≥35.5 mm	GC<35.5 mm	GC≥35.5 mm
ASV<4	1	132	2	0
ASV=4	0	0	105	1

Table 3 One-way analysis of variance of amylose content (AC) and gel consistency (GC) of the $F_{2:3}$ population using marker genotypes as the groups

[a] There are two degrees of freedom associated with the test for each of the effects
[b] Marker from the distal end of chromosome 6 where the gene for Q-enzyme I is located
[c] Marker from the short arm of chromosome 2 where the gene for Q-enzyme III is located

Trait	Locus	Chr.	MS effect [a]	MS error	*df* error	*F*	*P*
AC	*Wx*	6	2596.2	11.2	233	231.9	0.000
	R565	6	2463.4	12.5	235	196.5	0.000
	R1952	6	2606.4	11.3	235	230.3	0.000
	G200	6	689.8	27.7	234	24.9	0.000
	G342[b]	6	15.7	33.4	234	0.5	0.624
	R712[c]	2	72.9	33.0	233	2.2	0.112
	R2632	1	53.1	33.1	235	1.6	0.203
GC	*Wx*	6	10722.7	77.0	233	139.3	0.000
	R565	6	10586.8	78.1	235	135.6	0.000
	R1952	6	10362.9	80.0	235	129.6	0.000
	G200	6	2250.6	149.2	234	15.1	0.000
	G342[b]	6	370.8	165.4	234	2.2	0.108
	R712[c]	2	173.2	167.4	233	1.0	0.357
	R2632	1	88.8	167.4	235	0.5	0.589

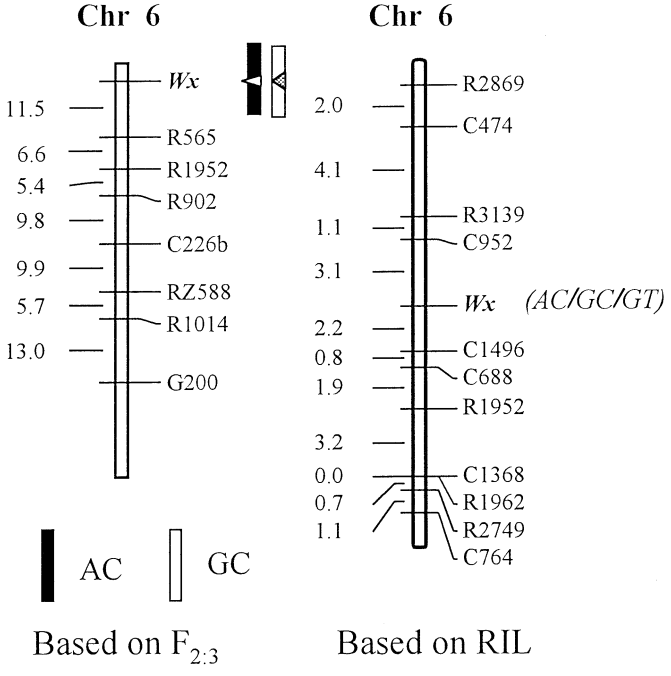

Fig. 3 Mapping of the genes controlling amylose content (*AC*), gel consistency (*GC*) and gelatinization temperature (*GT*) using two generations of the cross 'Zhenshan 97' × 'Minghui 63'. Map distances are expressed in cM on the left of the chromosome. The *bars* ($F_{2:3}$) indicate 1-LOD supporting intervals determined by the MAPMAKER/QTL analysis, and the *small triangles* indicate the peaks of the LOD contours

Fine resolution of the gene(s) for these traits using the RIL population

To obtain a fine resolution of the locus for these traits, 12 molecular markers that cover a segment of 20 cM surrounding the *Wx* locus were assayed using the 241 lines of the RIL population (Fig. 3). The local map constructed from these markers agreed well with the map of Harushima et al. (1998).

The data produced using the three SSR markers based on the *Wx* sequences showed perfect agreement with each other. Only two lines were still segregating at this locus and all the remaining 239 lines were homozygous for either the 'Minghui 63' or 'Zhenshan 97' genotype. Of the 239 lines, 238 demonstrated perfect associations among the three traits, these lines showed either high AC/low GC/low GT or low AC/high GC/high GT. An exception occurred in only one line in which high AC and low GC were associated with an intermediate GT.

Thus, the linkage of AC, GC and GT with the *Wx* locus was perfect among the aforementioned 238 lines. All individuals homozygous for the 'Minghui 63' genotype at the *Wx* locus showed a low AC, a high GC and a high GT, while the reverse was the case for lines homozygous for the 'Zhenshan 97' genotype at this locus.

Discussion

Amylose content, gel consistency and gelling temperature are generally considered to be the three most important traits that determine the cooking and eating quality of rice. Our results clearly showed that segregation for each of these three traits is controlled by a single locus in 'Shanyou 63', the most widely grown hybrid in China. The most important finding of the present study is that all the three traits are controlled by the *Wx* locus or else by a genomic region tightly linked to this locus.

In a pervious study, Wang et al. (1995) demonstrated that AC in the endosperm of rice grains is determined by the splicing efficiency of the first intron of the *Wx* gene. The perfect association of these three traits in this popu-

lation suggested a number of possibilities regarding the molecular mechanisms of GC and GT. One possibility is that each of the traits is controlled by a biochemical process that has its own molecular basis. Alternatively, GC and/or GT are simply the results of AC, such that for a certain value of AC, there is a corresponding level of GC and/or GT.

It should be noted in this connection that the two complementary combinations of the three traits, i.e. high AC/hard GC/low GT and low AC/soft GC/high GT, observed in this study are only a small fraction of the many combinations that are possible among the three traits. In a survey of rice germplasm from a large number of rice-producing countries of the world, Juliano and Villareal (1993) observed all kinds of combinations among various levels of these three traits. This indicates that the value of one trait is not necessarily dependent on the values of the other traits, which implies that each of the traits should have its own molecular basis specifying expression. It is also possible that not all three traits are specified by a single locus in other rice material; cases may exist where other loci may be involved in specifying the expression of one or all of the three traits. Regardless of these possibilities, co-segregation of the three traits in this population indicates that the genetic information controlling the segregation of the other two traits in this cross should reside either within the *Wx* gene sequence or else in a region very tightly linked to this gene. Thus, co-segregation of the three traits in this population may have provided a very useful clue to studying the molecular bases of these quality traits. The first place to look for such determinants may be to compare the *Wx* gene sequences between the parents, since the entire sequence of the *Wx* gene is already available (Wang et al. 1990).

Our results clearly have important implications in rice breeding programs. It is known that the cooking and eating qualities of rice constitute major problems in many rice producing areas of the world. These problems are particularly severe in hybrids and early season rice varieties of the multiple cropping systems in China. The finding that all three traits are controlled by a single locus has provided a well-defined target for quality improvement, which is especially the case for 'Shanyou 63', the cross used in this study. In particular, the availability of closely linked markers on both sides of this locus will greatly facilitate the precise replacement of the *Wx* allele of the poor-quality parent using marker-assisted selection. Such a precise replacement is particularly important for the improvement of hybrid parents in order to maintain the high combining ability while improving the quality.

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