

Full Length Research Paper

Quantitative trait locus (QTL) analysis of percentage grains chalkiness using AFLP in rice (*Oryza sativa* L.)

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Chalkiness is a major concern in rice (*Oryza sativa* L.) breeding because it is one of the key factors in determining quality and price, which is a complicated quantitative trait and controlled by maternal, endosperm and cytoplasmic effects. In this study, we conducted grain chalkiness percentage quantitative trait locus (QTL) analysis using amplified fragment length polymorphism (AFLP) marker and bulked segregant analysis combination. The materials was an F₁₂ recombinant inbred line population from a cross between two indica varieties, the parents of Shanyou63, the most widely grown hybrid rice in China. Three QTLs of grain chalkiness percentage were mapped in chromosome 3, 6 and 8, which were named *qIPGC-3*, *qIPGC-6* and *qIPGC-8*, respectively. The conventional method of QTL analysis clearly confirmed the loci of grain chalkiness percentage. It demonstrated that, AFLP technique coupled with bulked segregant analysis (BSA) can allow us to identify the locus governing primordial germ cell (PGC) and to effectively determine QTLs chromosome location of this locus on the molecular linkage map of rice.

Key words: Rice, quantitative trait locus (QTL), percentage of grain chalkiness, amplified fragment length polymorphism (AFLP).

INTRODUCTION

Rice chalkiness is an important appearance quality of rice grain. It represents a major problem of rice production in many rice producing areas of the world, especially in hybrid rice production in China. For improvement of milling, eating and cooking quality, the endosperm of high quality rice varieties should be free of chalkiness, since chalky grains have a lower density of starch granules than

vitreous ones and are therefore, more prone to breakage during milling (Del Rosario et al., 1968). Also, chalkiness reduces the palatability of cooked rice, since both longitudinal and transverse cracks occur easily in chalky kernels when the grain is steamed or boiled (Nagato and Ebata, 1959).

As one quality of endosperm trait, it is difficult for breeders to improve rice grain chalkiness using conventional methods, due to a lack of discrete phenotypic segregation in the progeny. Thus, identification of quantitative trait loci involved in grain chalkiness and elucidation of its genetic control are necessary for development of marker-assisted selection (MAS) and pyramiding breeding strategies aimed at improving breeding efficiency.

Rice chalkiness is a complicated quantitative trait, which is controlled by maternal, endosperm and cytoplasmic effects. Chalkiness is determined mainly by grain chalkiness percentage (PGC), area of chalky endosperm (ACE) and degree of endosperm chalkiness (DEC). Inheritance of rice grain chalkiness can be more complicated (Pooni et al., 1992; Zhu and Weir, 1994; Mo,

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Abbreviations: AFLP, Amplified fragment length polymorphism; QTL, quantitative trait locus; PGC, percentage of grain chalkiness; ACE, area of chalky endosperm; DEC, degree of endosperm chalkiness; MAS, marker-assisted selection; RILs, recombination inbred lines; BSA, bulked segregant analysis; PCR, polymerase chain reaction; CTAB, cetyltrimethyl ammonium bromide; BLAST, Basic Local Alignment Search Tool; SSR, simple sequence repeats.

1995). Using different mapping populations, a lot of quantitative trait locus (QTL), were found (Koh et al., 1999; He et al., 1999; Tan et al., 2000; Li et al., 2004; Li, 2001; Wan et al., 2005; Huang, 2006), however, the regulation and reason of rice grain chalkiness creating in different materials, QTLs in different background were not revealed.

White-belly endosperm trait has been fine mapping in chromosome 7, thirteen predicted genes were confirmed (Zhou et al., 2009). *OsPPDKB*, starch synthase IIIa (*SSIIIa*) and *GW2*, also have pleiotropic effects on white-core endosperm in rice (Kang et al., 2005; Fujita et al., 2007; Nayeon et al., 2007). The former encodes pyruvate orthophosphate dikinase (PPDK), which contributes to the control of carbon flow into starch and lipid biosynthesis during grain filling (Kang et al., 2005). *SSIIIa* plays an important role in the elongation of amylopectin chains (Fujita et al., 2007). *GW2* mainly impact on grain size in fruit development. These achievement and intensive study would pave the way for revealing the manipulative network and formative mechanisms of rice chalkiness.

In the present study, amplified fragment length polymorphism (AFLP) markers and bulked segregant analysis (BSA) method were used for PGC QTL identification in *indica/indica* background. AFLP technique was a powerful, reliable, stable and rapid assay in molecular-marker screening (Ballvora et al., 1995; Thomas et al., 1995; Cervera et al., 1996). BSA is a powerful means for gene mapping, especially single recessive gene (Michelmore et al., 1991). The objectives of this study were to detect QTLs affecting PGC and developing new method of QTLs detection using AFLP and BSA integration. The QTLs of PGC gave chance of MAS assays for grain molecular breeding.

MATERIALS AND METHODS

Genetic materials

Recombination inbred line population were used as PGC genetic and QTL analysis, which were the cross of Zhenshan97 and Minghui63, the parents of Shanyou63, the best hybrid in rice production in China. A total of 525 F₁₂ recombinant inbred lines (RILs) derived from the 1030 F₂ plants by the single-seed descendent method were used.

Field experiment design and conduction

Zhenshan97, Minghui 63 and 525 RILs were grown in rice growing season in 2006 on the experimental farms of Guang Zhou Academy of Agricultural Sciences, Guang Zhou and the winter of 2007 and 2008 in Hainan, China. At maturity, after drying, grains were stored at room temperature for 3 months and then dehulled and scored for PGC and other traits.

Trait measurement

PGC of different lines were evaluated according to He et al. (1999) and NSPRC (1999). To separate chalky from vitreous grains, three

replicates of 100 grains per entry were assessed by visual assessment. The mean percentage of chalky grains represented the PGC score for each line.

Scanning electron microscope (SEM) analysis of rice grain

For SEM observation, kernels were broken transversely and the pieces were mounted onto 12 mm aluminum stubs and sputtered with gold on a BioRad Polaron sputter coater. Samples were viewed under S-2500 Hitachi SEM and examined using SEM at an accelerating voltage of 20 kV. Diameters of starch granules were estimated on the basis of the scale bar provided on the captured.

DNA preparation and PCR protocol

Rice total DNA was extracted from fresh leaves using the cetyltrimethyl ammonium bromide (CTAB) method (Doyle, 1991). Polymerase chain reaction (PCR) was performed following the techniques of Chen et al. (1997). Briefly, PCR was performed using the procedure of a total volume of 20 µl, which contained 10 ng template DNA, 0.2 µM of each primer, 50 µM of dNTPs, 0.5 unit of Taq polymerase and 2 µl of 10 × buffer with 1.5 mM MgCl₂. Thirty-five cycles were carried out, with an initial 3 min period at 94°C followed by cycles of 40 s at 94°C, 40 s at 55°C and 1 min at 72°C and a final 10 min period at 72 °C. PCR products were separated on 3% agarose gel.

Molecular marker screening

AFLP markers were used in surveying PGC QTL loci. AFLP analysis was conducted according to Jia et al. (2001). After development, the target AFLP fragments were recovered from the gel. The gel slice containing DNA fragments were excised, eluted with 400 µl of high salt buffer (containing 20% ethanol, 1 M LiCl, 10 mM Tris-HCl, pH 7.5) and then, incubated at room temperature for 24 h and at 65°C for 2 h. The DNA was precipitated and one quarter of the re-suspended DNA was re-amplified using the same primer combination and PCR conditions as that of the selective amplification. The re-amplified DNA fragment was purified using a gene clean kit and then, cloned to the pGEM-T easy vector (Promega). The special PGC related AFLP fragments were sequenced and then e-PCR was carried out by the Basic Local Alignment Search Tool (BLAST) alignment between the genome sequences of *Japonica* cv. Nipponbare and *indica* cv. 9311. Further AFLP fragments associated with PGC were mapped on the genome. A closer of SSR markers in this region were selected for confirming the PGC QTLs. Genomic sequence was obtained from the International Rice Genome Sequencing Project (IRGSP) (<http://www.rgp.dna.affrc.go.jp/IRGSP/index.html>). SSR markers were identified from the Gramene database (<http://www.gramene.org>).

Linkage map construction and QTL surveys

For mapping QTLs of grains chalkiness percentage, a total of 256 AFLP markers distributed at certain genetic distances across all 12 chromosomes were employed in the polymorphism assay of Zhenshan97, Minghui63, high PGC bulk of Zhenshan97/Minghui63 RILs and low PGC bulk of Zhenshan97/Minghui63. Equal amounts of DNA, which were isolated from ten low PGC plants or ten high PGC plants in the F₁₂ generation of Zhenshan97/Minghui63, were pooled for low PGC bulked and high PGC bulk (Michelmore et al., 1991).

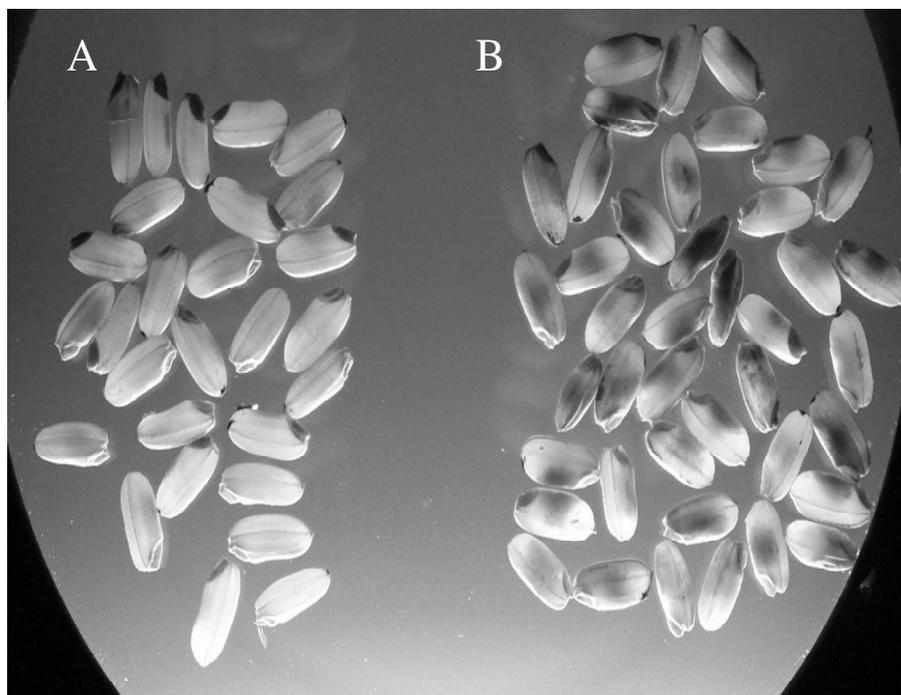


Figure 1. (A) Phenotype of low PGC grains (Minghui63); (B) phenotype of high PGC grains (Zhenshan97).

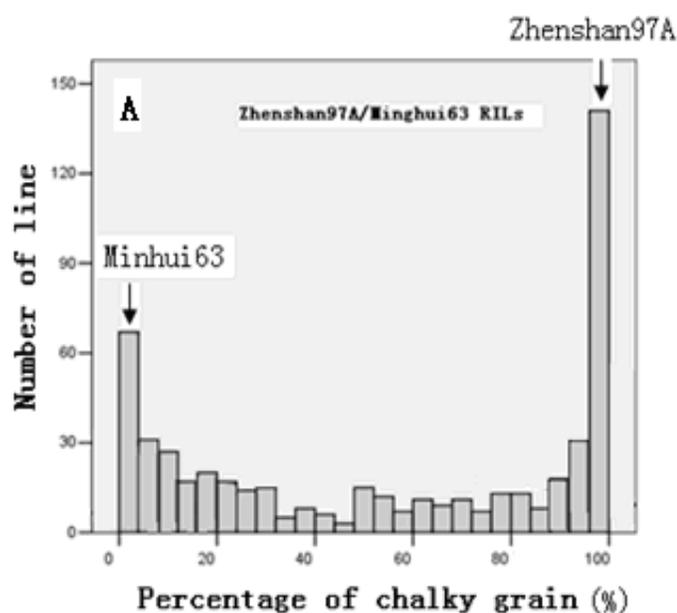


Figure 2. Frequency distributions of PGC in Zhenshan97/Minhui63 RILs and its parents.

The special AFLP fragments, which associated with PGC, were obtained by BSA method. According to the physical map and genetic map integrated analysis, the target gene loci were integrated into SSR genetic map (McCouch et al., 2002). Interval mapping was carried out to scan the whole genome for putative

QTLs controlling the PGC traits using MAPMAKER/QTL 1.1 with a LOD threshold of 3.0 (Lincoln et al., 1992). If two or more QTLs were detected in nearby regions, the QTL with the largest effect was fixed and the rest of the genome was re-scanned to confirm the existence of other QTLs. When a QTL was detected in an interval exceeding 20 cm in length in the linkage map, the existence of the QTL was confirmed using composite interval mapping (Zeng, 1994) as well as one-way analysis of variance using genotypes of the adjacent markers as the groups.

RESULTS

Characterization of chalkiness rice and genetic analysis of PGC

The chalky appearance is associated with the development of numerous air spaces between loosely packed starch granules and the resulting change in light reflection (Tashiro and Wardlaw, 1991; Lisle et al., 2000). Figure 1 showed the phenotype of rice grain in Zhenshan97 and Minghui63. The most grains in Zhenshan97 were chalky ones that had mainly white core with white area full of the grain; the PGC of Zhenshan97 was 95%. On the other hand, the PGC in Minghui63 was very low, only about 6.5%.

The distributions of grain chalkiness of Zhenshan97 (*Indica*)/Minghui63 (*Indica*) RIL populations are shown in Figure 2. Shanyou63 (Zhenshan97/Minghui63 F_1) had 87% PGC. In 525 RILs, 111 lines were 100% PGC, but 39 lines were 0% PGC and the same time, the range of

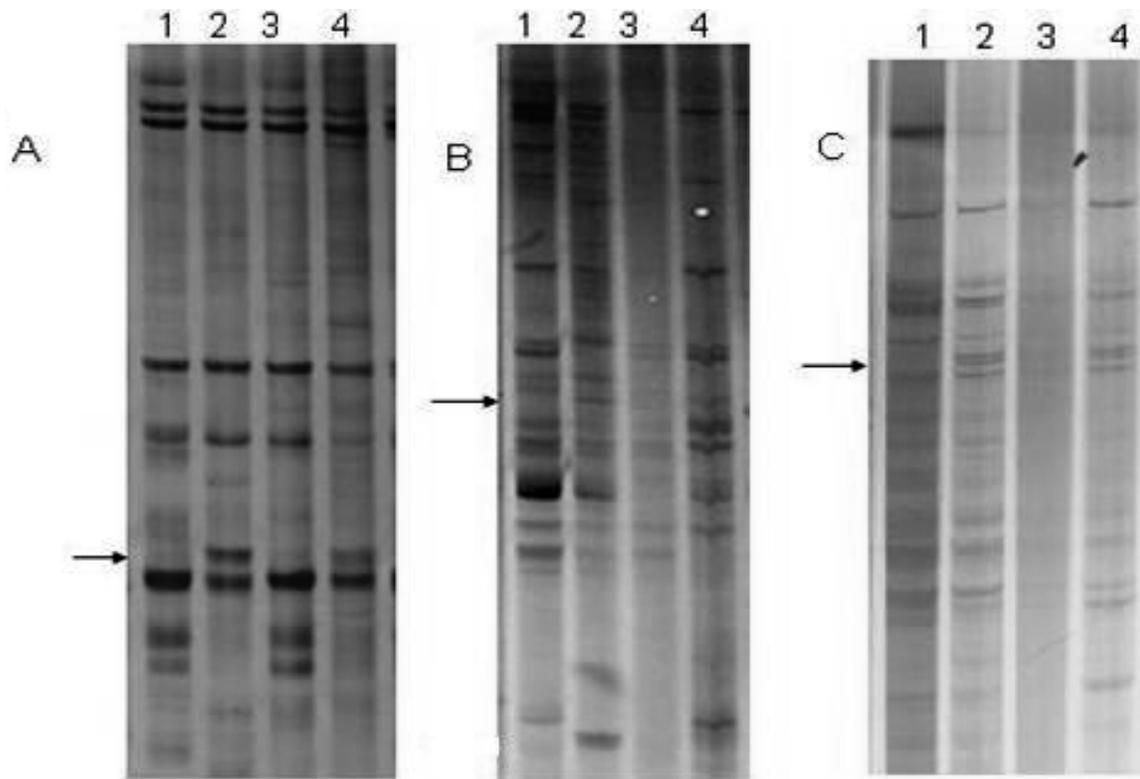


Figure 3. AFLP specific segment linked to rice percentage of chalky grain (A) AFLP results using primer E-ATA/M-CGT; (B) AFLP results using primer E-AAA/M-CTC; (C) AFLP results using primer E-AAA/M-CTA. 1, Minghui63; 2, Zhengshan97A; 3, low PGC bulk; 4, high PGC bulk. The arrow show linkage segments of percentage chalky grain in rice.

Table 1. Chromosomal location of AFLP segments linked to percentage of chalkiness grains.

No.	Name	AFLP primers	Location	Length of segment (bp)
1	T200	E-ATA/M-CGT	Chro.8 725709-725865	100-200
2	T216	E-AAA/M-CTC	Chro.3 23490806-23490932	100-200
3	T189	E-AAA/M-CTA	Chro.6 21086604-21086855	260

PGC distribution was 0 to 100%. The distribution mode of PGC showed continuous variation and was very different with an apparent bimodal distribution and uni-modal in RIL populations (Figure 2). A certain number of RILs segregated to their female parent transgressively, indicating PGC as a quantitative trait and the genetic effects of triploid endosperm and the cytoplasmic and diploid maternal were all of importance for PGC and chalkiness area (Shi et al., 2002).

Screening AFLP markers linked to PGC

Of the 256 AFLP markers produced, polymorphic bands between ZhenShan97 and Minghui63 were tested. These effective polymorphic markers were used for BSA analysis of PGC, which distributed across all of the 12

chromosomes. By bulk analysis, ZhenShan 97 and high PGC bulk both had positive band (the arrow showed) in primer E-ATA/M-CGT, Minghui63 and low PGC bulk had null band. But, it was null band in ZhenShan97 and high PGC bulk, positive band (the arrow showed) in Minghui63 and low PGC bulk using E-AAA/M-CTC and E-AAA/M-CTA (Figure 3). After that, the AFLP fragments which associated with PGC, were purified, cloned, sequenced and BLASTed, three loci were detected by this method, located on chromosomes 3, 6 and 8, respectively (Table 1).

QTLs for PGC

According to the information of e-PCR, some simple sequence repeats (SSR) markers were screened for

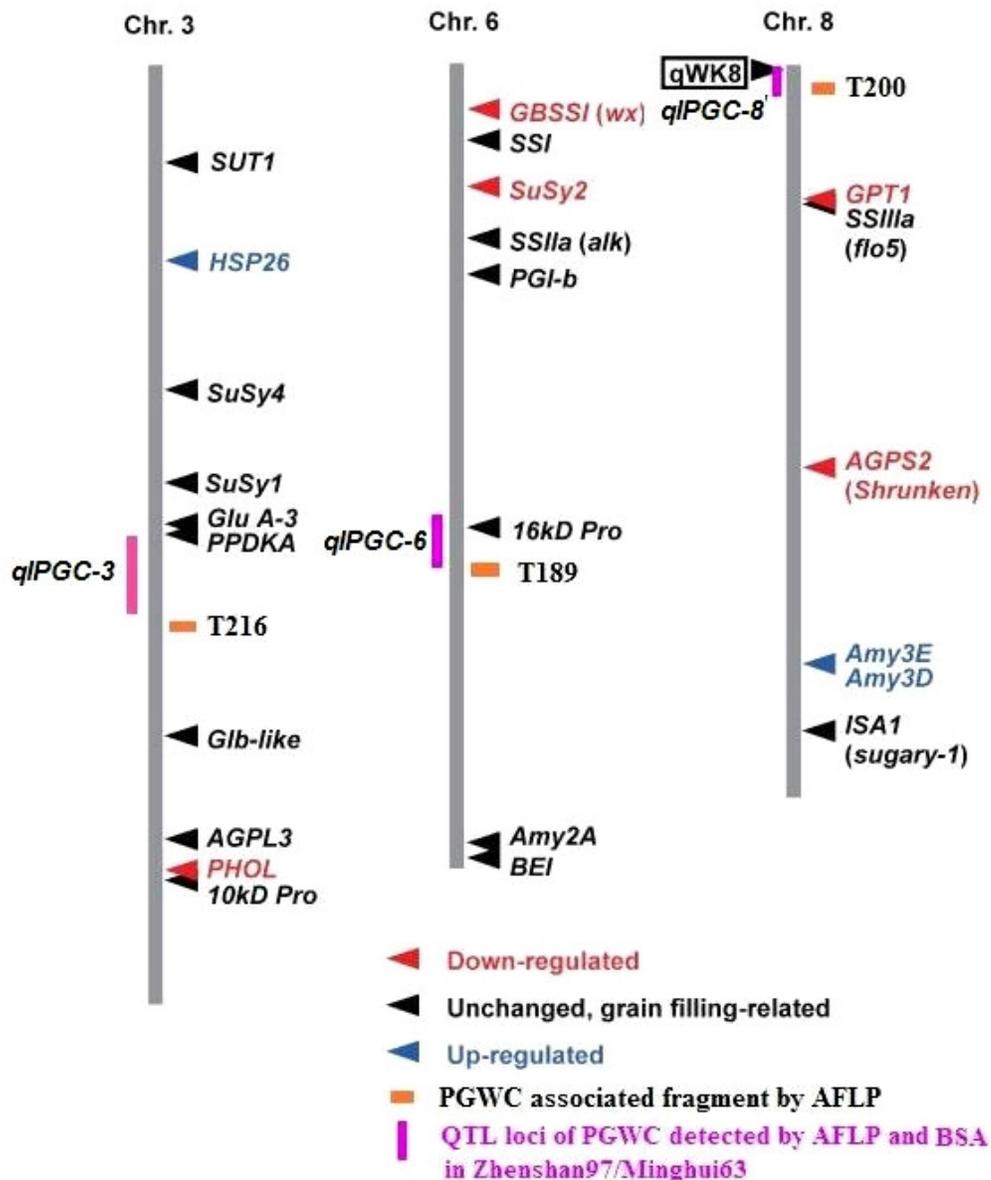


Figure 4. Comparison of PGC QTL detected in Zhenshan97/Minghui63 RILs using AFLP with filling related genes in rice. Up-regulated, unchanged and down-regulated genes related to grain filling metabolism, whose expression has been up-regulated, unchanged and down-regulated by high temperature, respectively. And they were indicated in blue, black and red, respectively. The associated fragment location of PGC, which was detected by AFLP were showed by pink bar. T216, T189, T200 were specific fragment segment linked to PGC, which was the AFLP primer E-AAA/M-CTC, E-AAA/M-CTA and E-ATA/M-CGT amplification in Zhenshan97/Minghui63 RILs, respectively.

confirming the PGC QTLs. Individual linkage analysis showed those three special AFLP markers linking with PGC, these loci were named *qIPGC-3*, *qIPGC-6* and *qIPGC-8*, respectively (Figure 4). Thus, three QTLs were detected for PGC in Zhenshan 97(*Indica*)/Minghui63 (*Indica*) RILs by AFLP and BSA combination.

Subsequently, QTL analysis was conducted with the

RIL progenies of Zhenshan97/ Minghui63 (Figure 4). Some SSR markers closed with these loci, were used confirming these PGC gene loci (data not show). The results indicated that, *qIPGC-3*, *qIPGC-6* and *qIPGC-8* were QTLs of PGC. According to the integrated analysis of genetic map with the starch biosynthesis genes, three QTLs were closed with starch biosynthesis related genes

Table 2. Chromosomal location of grain filling-related genes in chromosome 3, 6 and 8.

Enzyme/property	Gene name	Accession no.	Chromosome
Starch synthase	<i>GBSSI(wx)</i>	AK070431	6
	<i>SSI</i>	AK109458	6
	<i>SSIIa(alk)</i>	AK101978	6
	<i>SSIIIa</i>	AK061604	8
Branching enzyme	<i>BEI</i>	AK065121	6
Debranching enzyme	<i>ISA1(sugary-1)</i>	AB093426	8
α -Amylase	<i>Amy2A</i>	AK059671	6
	<i>Amy3D</i>	AK119761	8
	<i>Amy3E</i>	AK064300	8
ADP-Glc pyrophosphorylase	<i>AGPL3</i>	AK069296	3
	<i>AGPS2(shrunken)</i>	AK103906	8
Sucrose synthase	<i>SuSy1</i>	AK100334	3
	<i>Susy2</i>	AK072074	6
Phospho-Glc isomerase	<i>PG1-b</i>	AK068236	6
Sucrose transporter	<i>SUT1</i>	AK100027	3
Glc-phosphate transporter	<i>GPT1</i>	AK060577	8
Starch phosphorylase	<i>PHOL</i>	AK063766	3
Pyruvate orthophosphate dikinase	<i>PPDKA</i>	AK065739	3
Storage proteins prolamin	10kD <i>Pro</i>	AK108254	3
	16kD <i>Pro</i>	AK107785	6
Storage proteins glutelin	<i>Glu A-3</i>	AK107271	3
Storage proteins globulin	<i>Glb-like</i>	AK105347	3
Protein disulfide isomerase Heat shock protein	<i>HSP26</i>	AK063618	3

(Figure 4, Table 2). Among them, *qIPGC-3* was closed with *PPDKA* and *Glu A-3* and *qIPGC-6* was closed with 16 kD *Pro*. *qIPGC-8* was consistent with chalkiness QTL *qWK8* (Tabata et al., 2007; Yamakawa et al., 2007).

DISCUSSION

Yield and quality are two major subjects for many rice breeding programs, but grain yield and quality are both controlled by QTLs showing continuous phenotypic variation in rice progeny (Yano and Sasaki, 1997). The formation of rice chalkiness proves to be a complicated physiological process and tightly relate to "source-sink" of rice, dynamics of grain filling, biosynthesis and accumulation of starch in endosperm. The general picture that emerged from the genetic analyses of grain chalkiness showed that PGC was controlled by QTL. QTL identification is important for the illumination of grain chalkiness molecular mechanism.

In this study, we reported three QTLs of PGC, *qIPGC-3*,

qIPGC-6 and *qIPGC-8*, which were located in chromosome 3, 6 and 8, respectively. Among them, *qIPGC-8* on chromosome 8 was consistent with the reported PGC QTL with Tabata et al. (2007), and the consistency of *qIPGC-3* and *qIPGC-6* with high temperature-responsive genes in grain filling were consisted (Yamakawa et al., 2008). The results clearly demonstrated that, QTLs of PGC were likely to be controlled by "source-sink" gene.

The integration of molecular and traditional breeding was employed to demonstrate the application of the identified QTL in rice quality improvement and yield improvement. Based on this study achievement, breeding by design for pyramiding breeding of high quality, high yield and broad adaptability were carried out. Further, some lines were pitched on, which had high quality (such as low chalkiness grain) aggregation (data not show). It was a useful way for improving the appearance quality of different hybrid, especially to reduce chalkiness of the endosperm and at the same time, not to affect the yield.

At present in breeding practice, decrease of rice chalkiness has become one of the main aims in rice quality

breeding, especially for indica rice. This is the first report on mapping QTL using AFLP and BSA combination. The results clearly demonstrated that, it allowed us to identify the locus governing PGC and to effectively determine the chromosome location of QTL locus on molecular linkage map. Three polymorphic gene loci amplified from 256 primer combinations also showed high efficiency of the AFLP technique in QTL analysis.

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