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Breeding of a target genotype variety based on identified chalkiness marker-QTL associations in rice (*Oryza sativa* L.)

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ABSTRACT. The aim of this study was to breed a target genotype variety based on the identified chalkiness marker-QTL (quantitative trait locus) associations in rice. First, a permanent mapping population of rice that consisted of 525 recombinant inbred lines (RILs), which were derived from Zhenshan 97/Minghui 63, was used to identify QTLs with additive effects for rice quantitative traits and percentage of grain chalkiness (PGC). Subsequently, based on the identified QTLs in rice, the molecular marker 68923-PGC was selected to screen the low chalkiness rice line. Then, using the integration of molecular marker breeding and traditional breeding, we analyzed the genotype and phenotype of inbred lines from 525 RILs; we identified one rice variety with particularly high yields, good taste, and broad adaptability. The new variety was temporarily named

RIL10, which was a high quality, high yield, and broadly adaptable variety, and it is predominantly a feature that has contributed to its geographical adaptability, which would be planted from 35°E to 18°E in Chinain China, where 2/3 of rice production occurs. RIL10 was a marker-assisted selection breeding achievement for producing a high quality, high yield, and broadly adaptable rice variety.

Key words: Rice; Breeding by design; Chalkiness; QTLs; Molecular marker; Target genotype variety

INTRODUCTION

The goal of breeding by design can be reached by following a three-step approach (Peleman and Van der Voort, 2003): " 1) mapping loci involved in all agronomic relevant traits, 2) assessment of the allelic variation at those loci, and 3) breeding by design." Recent advances in sequenced rice genome information have made it possible to utilize phenotypic mutants to characterize relevant genes at the molecular level and reveal their functions. To date, 1698 genes have been reported for mutant or variant phenotypes were listed (Nori Kurata et al., 2005; Chen H et al., 2013). All of these achievements made it increasingly possible to practice breeding by design.

In practice, quantitative trait locus (QTL) pyramiding to combine loci for grain number and plant height in the same genetic background generated lines that exhibited both beneficial traits; these results demonstrated a strategy for tailor-made crop improvement (Ashikari et al., 2005). Another possible breeding strategy was able to increase grain length, decreasing grain width, reducing chalkiness of the endosperm, and, at the same time, not affect yield (Tan et al., 2000). Furthermore, a feasible strategy to enhance the efficiency in molecular breeding is simulation breeding (Wang et al., 2007).

Grain chalkiness is an important quality component of rice, as it has a profound influence on eating and milling qualities; in particular, it reduces the palatability of the cooked product (Cheng et al., 2005). Grain chalkiness is quantitatively inherited and controlled by effects of polygenes that are greatly modified by environments. Some QTLs associated with chalky grain have been identified (Li et al., 2003; Zhou et al., 2009; Liu et al., 2011; Liu et al., 2012). QTL inheritance was shown to be complex (Shi et al., 2002); therefore, if marker-assisted selection (MAS) strategies are applied for (or against) this trait, it is necessary to identify and mark the major QTL involved in trait determination. Several QTLs that control percentage of grains with chalkiness were identified on chromosomes 1, 5-10, and 12 (He et al., 1999; Koh et al., 1999; Tan et al., 2000; Li et al., 2004; Huang, 2006; Zhou et al., 2009). These accomplishments greatly facilitated breeding design of target genotypes based on the identified chalkiness marker-QTL associations in rice (*Oryza sativa* L.).

The objectives of this study were to detect the QTL mapping dataset for percentage of grain chalkiness (PGC) of rice and utilize the identified marker-QTL associations in designing a breeding methodology to produce rice inbred lines with low PGC and ideal agronomic traits. These results will provide a practicable illustration of crop improvement through design-breeding approach.

MATERIAL AND METHODS

Genetic materials

The genetic materials used in this study included Zhengshan 97 and Minghui 63, the parents of Shanyou 63, which is one of the best hybrids with regard to rice production in China, and 525 F_{12} recombinant inbred lines (RILs) were derived from 1030 F_2 plants by the single-seed descendent method. Forty-seven inbred lines selected from the 525 RILs through the design-breeding approach have been employed as an elite line used for molecular breeding.

Field experiment design and conduction

Zhengshan 97, Minghui 63, and their 525 RILs were grown in rice growing season in 2006 on the experimental farms of Guangzhou Academy of Agricultural Sciences and the winter of 2007 and 2008 in Hainan, China. Each plot consisted of four rows of 10 plants each, arranged in a randomized block design with three replicates. At maturity, each RIL, inbred line, and two parents were harvested in bulk. After drying, grains were stored at room temperature for 3 months, and then dehulled and scored for PGC and other traits.

Trait measurement

PGC was evaluated according to He et al. (1999) and NSPRC (1999). To separate chalky-form vitreous grains, three replicates of 100 grains per entry were visually assessed. The mean percentage of chalky grains represented the PGC score for that line. Other traits were measured according to Yu et al. (1997).

Molecular-marker screening

Amplified fragment length polymorphism (AFLP) analysis was conducted according to Jia et al. (2001). The AFLP fragments, which were cloned in the pUCm-T easy vector system, were then sequenced. Further, e-polymerase chain reaction (PCR) was carried out by BLASTN alignment between the genome sequences of *Oryza sativa* ssp. *japonica* 'Nipponbare' and ssp. *indica* '9311'. After, the specific fragments were mapped that were associated with percentage of chalky grain, and twelve SSR markers in this region were selected for fine mapping. The genomic sequence was obtained from the International Rice Genome Sequencing Project (IRGSP; http://www.rgp.dna.affrc.go.jp/E/IRGSP/index.html).

SSR markers were identified from the Gramene database (http://www.gramene.org). PCR was performed using a total volume of 20 µl, which contained 10 ng template DNA, 0.2 µM each primer, 50 µM dNTPs, 0.5 U Taq polymerase, and 2 µl 10X 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% agrose gel.

The closely linkage with PGC primer sequence of 68923-PGC, which reverse primer sequence (5'-3') was CTCGTATAGTAACTTGAGACG and the forward sequence (5'-3') was CCAAACAAGTTCCCACAATAG.

RESULTS

PGC genotype in RILs

In the bulked segregant analysis (Michelmore et al., 1991), 110 SSR primers were used to locate PGC controlling loci, and a total of five PGC QTLs were found using SSR marker screening (Liu, unpublished results). Among them, *PGC10* was mapped on the chromosome 10 between markers RM1873 and 68923-PGC; the DNA sequence between is approximately 880 kb. Using 68923-PGC primers, PCRs were performed using DNA samples from the two parents, eight individual plants with PGC >90%, and 10 individual plants with PGC <10%. The results show that the fragment amplified by 68923-PGC primers is highly associated with high PCG (Figure 1). Thus, the SSR marker 68923-PGC could be used in MAS of *PGC10* and breeding by design for high/low PCG using Zhengshan 97A, Minghui 63, or other lines with the same genetic background as a hybrid rice parent.

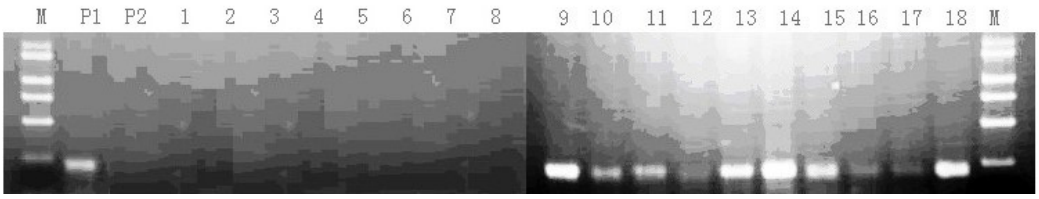

Figure 1. Progeny analysis with the microsatellite marker 68923-PGC. M, DL2000 marker; P1, Minghui 63; P2, Zhengshan 97A; 1-8, RILs that have 0% chalky grains; 9-18, RILs that have 100% chalky grain.

However, the PGC genetics are complicated; therefore, in the molecular breeding process, multiple factors should be taken into account. Additionally, the *PGC10* locus can only explain 10.5% of the variation. Therefore, 12 markers became reference points for design breeding, and these 12 markers were closely associated with *PGC5*, *PGC8-2*, and *PGC8-3*, which accounted for 25.5, 15.4, and 10.9% of the explained variation, respectively (Table 1). These integrated genotype combinations in PGC or other complicated traits are compulsory for molecular breeding, because quantitative traits are so intricate that no quantitative trait was completely associated with one genotype.

Table 1. Amplification results of PCR marker in some ZhenShan97A/Minghui63 RILs with 0 or 100 percent of chalky grain.

No	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
PGC (%)	0	0	0	0	0	0	0	0	0	0	100	100	100	100	100	100	100	100	100	100
cet200 (chro.8)	M	M	M	M	M	Z	Z	Z	M	M	Z	Z	Z	Z	Z	Z	Z	Z	M	M
RM25 (chro.8)	M	M	M	M	M	M	M	Z	M	M	Z	Z	Z	Z	Z	Z	Z	M	Z	Z
RM3395 (chro.8)	M	M	M	Z	Z	M	M	M	M	M	Z	Z	Z	Z	Z	Z	Z	Z	M	M
RM7285 (chro.8)	M	M	M	M	Z	Z	M	M	M	M	Z	Z	M	Z	M	M	Z	Z	M	M
RM210 (chro.8)	M	M	M	M	Z	M	Z	M	Z	M	Z	M	Z	Z	M	M	Z	Z	M	Z
RM447 (chro.8)	M	M	M	M	Z	M	Z	M	M	Z	Z	M	Z	Z	M	M	Z	Z	Z	Z
RM281 (chro.8)	M	M	M	M	M	Z	M	Z	Z	M	Z	Z	Z	M	Z	Z	M	M	Z	Z
RM289 (chro.5)	M	M	M	M	M	M	M	Z	M	M	Z	Z	Z	Z	M	M	Z	Z	Z	Z
RM3437 (chro.5)	M	M	Z	M	M	M	M	M	M	M	Z	Z	M	Z	Z	Z	M	M	Z	Z
RM1873 (chro.10)	Z	Z	Z	Z	M	Z	Z	Z	Z	Z	M	Z	Z	M	Z	M	M	M	M	Z
68923-PGC (chro.10)	Z	Z	Z	Z	Z	Z	Z	Z	Z	Z	M	M	M	M	M	M	M	M	M	M
RM304 (chro.10)	Z	Z	Z	Z	M	Z	Z	Z	Z	Z	M	Z	Z	M	M	M	M	M	M	M
Cet189 (chro.6)	M	Z	M	Z	M	Z	M	M	M	M	M	M	Z	M	Z	Z	Z	Z	Z	Z
RM5551 (chro.3)	Z	Z	Z	Z	M	M	M	Z	M	M	M	M	Z	Z	Z	Z	Z	Z	M	Z

Remark: M indicates that the genotype of this recombination inbred line is the same with Minghui63; Z indicates that the genotype of this line is thesame with Zhenshan97A.

At the same time, 256 primer combinations were employed, and some polymorphic AFLP bands were obtained. The AFLP fragments that were associated with PGC, were purified, cloned, sequenced, and expression-PCR (e-PCR) on the position of the chromosome. By bulk analysis, Zhenshan 97 and high PGC individuals all had positive bands (arrow in Figure 2A) in primer E-ATA/M-CGT. However, Minghui 63 and low PGC individuals had null bands (Figure 2A). Furthermore, linkage analysis was carried out in individuals, which showed that this marker is close to the PGC gene (Figure 2B). This fragment was purified, cloned, sequenced, and blasted; the results showed that this fragment was located in chromosome 6 (Figure 3A). Three loci were detected by this method, including *PGC3*, *PGC6*, and *PGC8-1*, and some SSR markers associated with *PGC8-1* (cet200 and RM25; Table 1) were confirmed.

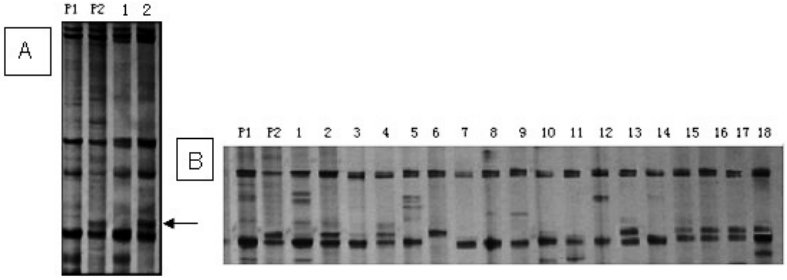

Figure 2. AFLP-specific segment linked and progeny analysis with E-ATA/M-CGT **A.** AFLP-specific segment linked to rice percentage of chalky grain by the AFLP primer E-ATA/M-CGT (P1, Minghui 63; P2, Zhengshan 97A; 1, low percentage of chalky grain bulk in rice; and 2, high percentage of chalky grain bulk in rice. The arrows show linkage segments of percentage of chalky grains in rice.). **B.** Amplification of some Zhenshan 97A/Minghui 63 RILs using the AFLP marker E-ATA/M-CGT (P1, Minghui 63; P2, Zhenshan 97A; 1-10, RILs that have 0% chalky grains; 11-16, RILs that have 100% chalky grains).

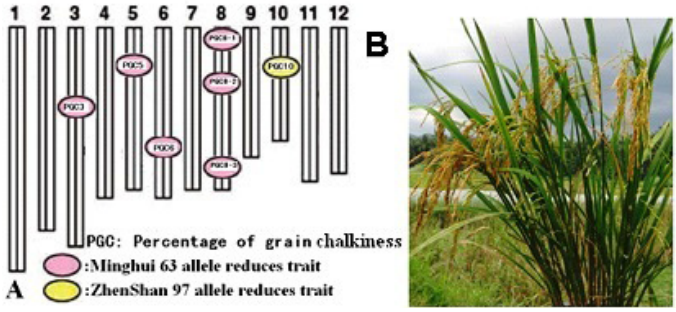

Figure 3. Genotype and phenotype in ideal line, RIL10. **A.** QTL map of PGC in inbred line RIL10. **B.** Gross morphology of inbred line RIL10 at maturity.

Breeding of the target genotype line

Using selected markers (Table 1), genotypes of the 525 RILs with PGC correlative loci were analyzed. Twenty RILs (RIL1-RIL20) were selected, and their genotype and agronomic traits profiles were investigated (Tables 1 and 2). Low PGC RILs were not identical to Minhui 63 with regard to all of the PGC loci genotype, but they were similar to the primary Minhui 63 genotype. Because Minhui 63's PGC was 6.5%, if the RILs' PGC reached 0%, the RILs needed some alleles from Zhenshan 97, which reduced the PGC.

Table 2. Performances on appearance quality and yield related traits of 20 RILs.

No.	GL (cm)	GW (cm)	GT (cm)	LWR	GWT g/1,000	PGC (%)	Yield (t/ha)	SST (%)	Brown rice (%)	Head rice (%)	Plant height (cm)	Tillers/plant
1	0.84	0.23	0.19	3.65	18.50	0.00	4.73	99.00	75.40	62.10	90.00	11.70
2	0.84	0.26	0.23	3.23	22.00	0.00	3.35	95.00	70.60	65.50	102.30	13.00
3	0.85	0.21	0.20	4.05	16.50	0.50	4.69	99.00	75.90	70.80	92.70	8.70
4	0.82	0.28	0.21	2.93	22.50	0.20	8.15	98.00	72.20	68.90	90.00	14.00
5	0.89	0.26	0.22	3.42	25.00	5.40	5.27	97.00	73.90	70.60	102.70	10.00
6	0.95	0.27	0.22	3.52	18.00	1.00	3.87	92.00	48.00	39.30	80.30	16.00
7	0.97	0.25	0.19	3.88	24.50	2.00	3.61	94.00	75.20	72.10	96.30	11.70
8	0.84	0.26	0.22	3.23	20.50	0.00	3.27	89.00	71.00	65.40	101.30	17.30
9	0.87	0.20	0.20	4.35	16.00	0.00	3.89	88.00	72.40	70.50	93.00	9.00
10	0.80	0.24	0.22	3.33	18.00	0.00	7.50	98.00	71.10	70.90	102.70	11.30
11	0.77	0.31	0.19	2.48	27.00	86.40	5.96	95.00	77.50	72.90	100.30	6.70
12	0.80	0.21	0.20	3.81	25.00	84.00	4.12	98.00	71.90	56.90	98.00	7.30
13	0.87	0.32	0.22	2.72	18.00	92.70	2.91	99.00	61.90	50.00	108.00	8.30
14	0.82	0.25	0.23	3.28	25.50	73.80	4.12	84.30	75.70	59.30	84.00	8.70
15	0.83	0.26	0.22	3.19	24.00	85.50	4.02	91.00	78.80	71.00	98.70	8.70
16	0.78	0.22	0.21	3.55	21.50	80.20	3.80	100.00	70.60	62.50	83.70	9.00
17	0.78	0.31	0.24	2.52	24.50	77.40	4.72	96.00	64.20	54.30	88.70	8.70
18	0.82	0.27	0.22	3.04	24.00	87.50	5.12	91.00	78.00	71.00	98.70	9.70
19	0.86	0.27	0.21	3.19	27.00	82.70	5.67	95.00	71.00	65.00	82.00	11.00
20	0.84	0.31	0.20	2.70	26.00	76.00	6.05	89.00	74.00	72.50	91.00	10.60

The breeding goal is to enhance grain yield, and pyramiding QTLs to combine high yield and high quality loci in generated lines with the same genetic background exhibited beneficial traits. Ultimately, the breeding of RIL10 (a line that is high quality and high yield) can provide tailor-made crop improvement (Figure 3B). In this experiment, some QTLs associated with PGC were identified. To improve milling, eating, and cooking quality, the endosperm of high quality rice varieties should be free of chalkiness, because chalky grains have a lower density of starch granules than vitreous grains and are therefore more prone to breakage during milling (Del Rosario et al., 1968). Furthermore, because both longitudinal and transverse cracks occur easily in chalky kernels when the grain is steamed or boiled, chalkiness reduces the palatability of cooked rice (Nagato and Ebata, 1959). Therefore, the low PGC alleles and null PGC lines were selected first. Then, other components of important agronomic traits were ideally suited for application to a practical breeding program.

The three steps of breeding by design include 1) selection of chalkiness genotype; 2) selection of suitable traits of plant high, yield/panicle, tiller/plant; and 3) other agronomic important traits selecting. In breeding practices, an ideal line can be created by design breeding approach that combines high quality, high yield, and other appropriate characterizations. The elite RIL10 line encompassed both ideal genotype and gross morphology. The important RIL10 traits were suitable for the breeding selection process based on the evaluation index, which included percentage of chalkiness grain (0%), plant height (102.7 cM), tiller/plant (11.3), yield/panical (3.51 g), grain length (0.8 cM), grain width (0.24 cM), grain thickness (0.22 cM), and length-width ratio of grain (3.33). Using this approach, a desirable variety was successfully bred by combining important QTL MAS and common genetic background selection.

DISCUSSION

Achieving the target genotype by optimum selection strategies

The QTLon chromosome 5 was a major locus that explained 87.2% of the variation, and the other QTL played only a minor role in specifying this trait (Tan et al., 2000). In this case, alleles from Zhenshan 97 increased the opacity of the grains. Fujita et al. (2007) described a starch synthase III (*SSIIIa*) mutant with white-core endosperm, in which the structure and components of the endosperm starch were profoundly altered. Moreover, Ryoo et al. (2007) found that *SSIIIa* causes white-core floury endosperm in rice. Zhou et al. (2009) described *qPGWC-7* as the first fine-mapped gene for white-belly endosperm in rice, which can explain 60.6% of phenotypic variation in their population. In this study, seven QTLs were used for selecting the targeting genotype. We succeeded in breeding by design and obtained an inbred line that produces ideal gross agronomic traits; an inbred line in particular produces high yield and high quality rice. This line has optimum duration, remarkably effective tillers, ideal grains weight, high yield, beautiful grain shape, good head rice recovery, acceptable chalkiness, resistance to leaf folder, and preferred taste. RIL10 can be qualified as the super market variety in China; the taste is almost as good as that preferred by Chinese consumers, and the appearance is as good as the best *indica* rice.

Identification of agronomic important QTLs and pyramiding of such QTLs were presented a useful strategy for efficient crop breeding. However, crop breeding is a complicated process. In practice, many elite lines breeding experience revealed that breeding is a cumulative process that includes many steps. In fact, other factors, such as grain quality, resistance to pests, and other adversities, must also be taken into account. Besides growing duration and seed-setting ability, numerous other crucial traits directly or indirectly relate to yield and quality. Many traits are

often judged, including rapid growth and development, aerial spacing of tillers, tolerance to heavy fertilizer application, resistance to lodging, leaf function at the late-growing stage, senescence of the spikelet rachilla and axial branch, leaf and grain color change at the ripening stage, viability of the root system, high photosynthetic efficiency, the relationship between source and sink as well as adaptability, rice grain quality includes the milling, appearance, cooking and nutritional qualities, and grain qualities (Yang et al., 1996).

Improving chalkiness trait using “hidden diversity”

Some studies have demonstrated “hidden diversity.” This term means that, despite the complex genetics and diverse physiological mechanisms underlying the abiotic stress tolerances, introgression of genes from a diverse source of donors into elite genetic backgrounds through breeding and efficient selection (careful screening under severe stress) is a powerful way to exploit this hidden diversity to improve abiotic stress tolerances of rice (Li et al., 2005; Ali et al., 2006). Genetic control of grain chalkiness demonstrated that transgressive inheritance often occurs in many combinations (Tan et al., 2000; Zhou et al., 2009). In this study, we found 39 inbred lines whose chalkiness grains are null. These studies revealed that two parents both have hidden diversity.

Application of QTLs for molecular breeding

Discovering useful genes, improving agricultural traits hidden in the plant genome, and applying these findings to crop breeding will pave the way for breeding by design. An elite line has a combination of super traits. For example, Shanyou 63 is the best hybrid in China and was produced by crossing Zhenshan 97 with Minghui 63. 131 rice restoring lines germplasm and 249 elite hybrid were obtained from breeding programs for Minghui 63 in China. In molecular breeding, breeders highlight the importance of genetic networks that underlie the complex phenotypes in rice; revealing these networks ultimately lead to a more complete understanding of genetic and molecular bases of quantitative trait variation in rice. For improving the plant high character of rice, the quantitative trait locus of *sd-1* gene were used. Although the QTL *sd-1* accounts for 59.8% of the total variation, a breeder can control the plant height by utilizing *sd-1* and integrating other QTLs that affect plant height. Rice breeding in Japan optimized eating quality and plant height. Highly palatable rice was created by using Koshihikari as the donor parent. Here, we first carried out analysis on high quality QTLs (such as grains low in chalkiness) aggregation; then, some lines were included that had other desirable traits. Ultimately, new varieties were produced, and they integrated many favorable properties.

Conflicts of interest

The authors declare no conflict of interest.

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