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Molecular mapping of quantitative trait loci for grain chalkiness in rice (*Oryza sativa* L.)

P. R. Chandusingh[1], N. K. Singh[2], K. V. Prabhu, K. K. Vinod and A. K. Singh*

Division of Genetics, Indian Agricultural Research Institute, New Delhi 110 012; [1]Directorate of Seed Research, Mau 275101; [2]National Research Centre on Plant Biotechnology, New Delhi 110 012

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Abstract

Chalkiness of rice grain is an important but undesirable quality component, which has a profound influence on milling, cooking and eating qualities. Therefore, it is one of the key factors which determine the market value of rice grains. In this study, QTL mapping to determine the genomic regions governing percent grain with white core (PGWC) and chalkiness score (CS) was conducted using a population of 310 RILs, generated from the cross Pusa 1266/Jaya. Both the parents were extremely contrasting with Pusa 1266 with fully chalky and Jaya with translucent grains. RILs were evaluated at IARI, New Delhi during *Kharif* 2008 and 2009 and at RBGRC, Aduthurai during *Rabi* 2009-10. Several QTL signals could be detected by single marker analysis. Among them, two QTLs, one each for PGWC and CS, were prominently detected by composite interval mapping between a marker interval RM 6273-RM204 on the short arm of chromosome 6. The QTL for PGWC, *qPGWC6* was consistently detected during all the three seasons, explaining a phenotypic variation of 17.75%, 18.79% and 12.20% respectively. Similarly, the QTL for CS, *qCS6* was also consistent across three seasons, accounting for a phenotypic variation of 24.3%, 24.75% and 24.03% respectively. Co-localization of both these QTLs may be representing a single genomic region, strongly implying the presence of *Wx*^{b} locus in contributing to grain chalkiness of Pusa1266. The positive allele for the reduced grain chalkiness was contributed by Jaya. These results will be useful in fine mapping and validation of the identified QTLs.

Key words: Quantitative trait loci, percent grain with chalkiness score, simple sequence repeat, new plant type

Introduction

Rice is one of the major staple food crops which feeds more than half of the world's population. Although the preferences for rice grain characteristics vary with different consumer groups, quality rice varieties are preferred by consumers and producers' alike. Characterized as 'chalky', the opaque part of the milled rice grain rather than being translucent, is an undesirable quality parameter. Chalkiness disappears on cooking and has no effect on taste but it is associated with high degree of damage to the kernel during milling, which leads to high percentage broken grains and thus substantial reduction in head rice recovery [1]. Milled rice with more than 8% chalky kernels is generally not accepted in most world markets [2].

The chalky areas of the grain contains less densely packed starch granules which make them soft as compared to translucent areas. These areas are mechanically weak leading to breakage during milling. The degree of chalkiness varies greatly among cultivars and among environments within a cultivar. Based on opaqueness, chalkiness is classified into four categories based on position and amount of opaqueness in the milled rice grains such as, i) on the dorsal side of the grain (white belly), ii) on the ventral side (white back), iii) in the center (white center), and iv) pit left by the embryo (eye) [3]. The high temperature stress during rice grain filling leads not only to increased degree of chalkiness but also decreased grain-filling rate, decreased grain weight, reduced amylose content [4].

The inheritance of rice grain chalkiness has been studied by various researchers in the past. Reports on the inheritance of grain chalkiness ranges from white center and white belly as monogenic recessive traits (*wc, wb*) [5] to white belly as dominant trait [6] and to a multigenic system [7] interacting with environmental

*Corresponding author's e-mails: aks_gene@yahoo.com, aksingh021@rediffmail.com
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factors. In certain genotypes, chalky grains do not appear in any environment, while in some other degree of opacity vary between different environments, to the degree of complete translucency to complete chalkiness. In certain cases, grains from the same panicle may show different degree of chalkiness [8]. The quantitative inheritance appears to be more plausible mechanism in determining grain chalkiness [9, 10].

After the advent of molecular marker based technology [11], several QTL mapping experiments have been conducted in order to understand the genetic basis of this complex trait [9, 10, 12, 14-16]. More than 82 QTLs governing chalkiness related traits [13] including 30 QTLs for percentage grain with white core (PGWC) have been identified across the rice genome [14, 15]. Further, there are 12 QTLs for area of endosperm chalkiness (AEC) [15], 3 QTLs for basal white (BW) [16], 26 QTLs for degree of endosperm chalkiness (DEC) [14, 15] and 11 QTLs for white backed kernel (KW) [16]. In the present study, simple sequence repeat (SSR) markers were used for identifying QTLs governing chalkiness traits using 310 F_8 recombinant lines from the cross, Pusa1266/Jaya.

Materials and methods

A mapping population consisting of 310 F_8 RILs generated earlier in our lab [20] from a cross Pusa1266/Jaya was used for QTL mapping for PGWC and CS traits in the present study. Pusa1266 is a semi dwarf new plant type (NPT) rice genotype developed at Indian Agricultural Research Institute, New Delhi through *indica-japonica* hybridization involving multiple parents like Tainan 3, a tropical *japonica* variety, Xiangnuo 4, an aromatic glutinous *japonica* variety, IR72, a high yielding *indica* variety and wide compatibility donors such as N22, Dular and Gharbaran. It is characterized by high tillering, sturdy culm, dark green, erect leaves, high grain number per panicle and fully chalky grains. Jaya is a semi dwarf, high yielding *indica* rice variety with translucent white kernels.

Field experiment design and implementation

RILs were evaluated at Indian Agricultural Research Institute (IARI), New Delhi during *kharif* 2008 and *kharif* 2009; and at Rice Breeding and Genetics Research Centre (RBGRC), Aduthurai, Tamilnadu during the late samba (*Rabi*) season, 2009-10. The seedlings when 25 days old were transplanted in three rows with 20 single plants per row at spacing of 30 cm between rows and 15 cm between plants in augmented block design with five blocks with four checks including both the parents. The experiment received standard agronomic care.

Trait measurement

At maturity, each RIL was harvested in bulk, threshed and the grains were sun dried for 3 to 4 days to bring the moisture content to 12%. Approximately 150g sample of rough rice was taken and dehulled using a laboratory husker (Satake® THU35C testing husker). The brown rice was polished with the help of a laboratory mill (Satake® TM05C rice test mill). Full rice kernels were separated from each milled rice samples and two samples of 100 full grains each were randomly drawn to record data on PGWC and CS based on the visual observation as per method suggested by Indian Institute of Crop Processing Technology. PGWC was calculated as the proportion of chalky grains out of 100 for each RIL, averaged over two replications.

A visual rating of the chalky portion of the grain was used to quantify the per cent area of chalkiness which was then used to develop a CS. 100 grains were placed on black papersheet and number of grains with given per cent area of chalkiness as per scale (Table 1) were counted in each category. CS was calculated by using following formula,

$$CS = \Sigma nS/100$$

where, ‘n’ is the number of grains per each score category and ‘S’ is the score for corresponding category [17].

Parental polymorphism survey and genotyping of RILs

Table 1. Score scale based on per cent area of chalkiness

Score	% area of chalkiness
0	Nil (translucent)
1	1-10
5	10-20
7	25-50
9	$\geq$ 50

DNA was extracted from fresh leaves of parents and RILs by CTAB (Cetyl-trimethyl ammonium bromide) method as described by Prabhu *et al*. [18]. The PCR reactions consisted of 25 ng of genomic DNA, 0.2 U of *Taq* DNA polymerase (Bangalore Genei), 1X PCR

assay buffer with 1.5 mM $MgCl_2$, 12 ng each of forward and reverse primer and 200 µM of dNTP mix with a thermal cycling profile of initial denaturation at 94°C for 5 min followed by 35 cycles consisting of denaturation at 94°C for 40s, annealing at 55-60°C for 40s, extension at 72°C for 1 min and a final extension of 7 min at 72°C. The PCR amplified fragments were resolved on 3% Metaphor® agarose gel (Lonza, USA).

Genotypic data of the RILs with 162 polymorphic markers was made available from two previous studies in the lab [19, 20]. Additionally, 66 rice microsatellite (RM) series primers and hypervariable microsatellite (HvSSR) markers [21] along with 18 RM series markers linked to QTLs for PGWC and CS reported in earlier studies [14-16, 22] were used for polymorphism survey (Fig. 1). Since 12 of these markers were found polymorphic, the genotyping data of the RILs consisted of a total of 174 markers that provided genome wide coverage. Most of the marker loci followed expected Mendelian segregation (1:1), except for 42 marker loci distributed across chromosomes [23]. Hence, a total of 132 polymorphic markers were used for linkage map construction.

Construction of genetic linkage map and QTL analysis

Linkage map construction was carried out using MapMaker/Exp v3.0 [24] using genotype data obtained from 132 markers. Multi-point analyses conducted on different linkage groups did not link sixteen markers, therefore, a total of 116 polymorphic markers were used to construct a linkage map. The total map length was estimated to 1560.7cM with an average marker interval of 13.45cM.

QTL analysis was carried out using WinQTLcart v2.5 [25]. As the preliminary step, single marker analysis (SMA) was carried out using genotype data of all 132 markers and followed by composite interval mapping (CIM) as the final step. The phenotype data for each season was done independently. For CIM, LOD thresholds were computed separately for each traits by conducting 1000 permutations at a significance level of $p<0.05$. QTL peaks exceeding the threshold values were declared significant. Nomenclature for QTLs followed gene nomenclature system for rice [26].

Results and Discussion

Phenotyping of RILs for PGWC and CS

The data on PGWC and CS of parents and the RIL population are presented in Table 2. The grains of Pusa 1266 were completely chalky with PGWC 100% and CS of 9, while Jaya possessed comparatively low chalky grains with PGWC of 28% and CS of 0.64. Among the RILs, the PGWC ranged from 0 to 100% with 193 RILs scoring PGWC of 100% while 2 RILs scored PGWC of 0%, the remaining 115 RILs were found to have varied degree of PGWC. Transgressive segregation for PGWC was observed in 90 out of 310 RILs (29%), with PGWC score being less than the parent Jaya. Similarly, for CS, significant difference between parents was observed with Pusa 1266 and Jaya scoring 9 and 0.64%, respectively. Among the RILs, CS value ranged from 0 to 9, with 191 RILs possessing CS of 9 and 2 RILs possessing CS of 0, while remaining 117 RILs showed CS values in between. Transgressive segregation for CS was observed in 77 out of 310 RILs (24.3%), which showed CS less than the parent Jaya. Further, such deviations could be observed at both New Delhi and Aduthurai environments. Based on the skewed phenotypic distribution and transgressive segregation of PGWC and CS in the RIL population one would conclude that these traits are under polygenic control, with involvement of modifiers and/or with QTL-environment interaction. Similarly transgressive segregation was observed for yield and yield related traits [20, 27, 28], for spikelet setting density and for flag leaf length [20, 29] in earlier reports.

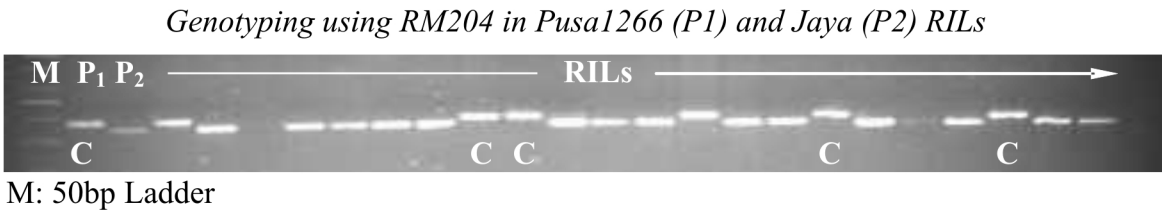

Fig. 1. Genotype profile of the marker RM204 among Pusa 1266 (P_1), Jaya (P_2) and RILs. 'C' indicates chalky genotypes

Table 2. Descriptive statistics and transgressive segregation (%) for PGWC and CS over three seasons

Characters	*Kharif* 2008 (New Delhi)		*Kharif* 2009 (New Delhi)		*Rabi* 2009-10 (Aduthurai)	
	PGWC	CS	PGWC	CS	PGWC	CS
Mean	61.2	4.5	68.3	4.8	69.2	4.8
Standard deviation	37.3	4.0	31.7	3.7	32.5	3.7
Maximum	100.0	9.0	100.0	9.0	100.0	9.0
Minimum	0.0	0.0	0.0	0.0	0.0	0.0
Pusa 1266	100.0	9.0	100.0	9.0	100.0	9.0
Jaya	28.0	0.4	30.0	0.5	36.0	1.0
No. of Transgressive segregants	90.0	55.0	53.0	38.0	68.0	72.0
Transgressive segregation (%)	29.0	17.7	17.1	12.3	21.9	23.2

PGWC, Percentage of grains with white core; CS, Chalkiness score

QTLs for PGWC and CS

Development of chalkiness in rice grains is a complex genetic phenomenon, which is greatly influenced by source-sink relationship, dynamics of grain filing, biosynthesis and accumulation of starch in endosperm. PGWC and CS are two important quantitative measurements of grain chalkiness. While PGWC measures the extent of chalky grains CS measures the degree of chalkiness in each of the kernels. Therefore, identification of QTLs for PGWC and CS can be of great help in marker assisted selection for reduced grain chalkiness in rice.

Single marker analysis of the marker trait segregation data among the RILs revealed several QTL signals, most of which were weak (Table 3). Although there were consistency of such signals among four markers (RM5310, RM6273, RM204 and RM201), across three seasons spread over two locations of New Delhi and Aduthurai, highly significant phenotype variation explained (PVE) values were exhibited only by two neighboring markers on chromosome 6, RM6273 and RM204. Further, CIM analysis resolved, one QTL for PGWC on chromosomes 6 was between marker interval RM6273-RM204 consistently across all three seasons (Table 4). The QTL, *qPGWC6* had a LOD score of 10.55, 11.71 and 22 and with corresponding PVE of 17.75%, 18.79% and 12.20% respectively (Fig. 2). The parent Jaya, which had lower values for PGWC and CS compared to Pusa1266, contributed alleles with decreased chalkiness effect at loci i.e. *qPGWC6.* Similarly the QTL detected for the trait CS, *qCS6* was consistently detected in all three seasons and irrespective of locations. The QTL

Table 3. Percent phenotypic variation (R^2) captured by significant QTL signals identified by single marker analysis

Marker	Chrom	Percent Grain with white core (PGWC)			Chalkiness score (CS)		
		2008 *kh*	2009 *kh*	2009-10 *ra*	2008 *kh*	2009 *kh*	2009-10 *ra*
RM5310	1	-	-	1.64*	2.12*	1.88*	2.11*
GNMS1289	3	1.33*	1.94*	1.82*	-	-	1.70*
RM3698	3	-	-	2.06**	-	-	-
RM6273	6	4.83***	4.64***	3.18***	5.61***	5.26***	6.42***
RM204	6	11.31***	13.86***	11.22***	13.75***	14.53***	13.26**
RM201	9	2.05*	1.97*	2.97**	1.83*	1.78*	3.19**
RM278	9	-	-	1.24*	-	-	-
GNMS3235	11	1.40*	-	-	-	-	-

*,**,***- significance at p<0.05, 0.01 and 0.001 respectively; Chrom, chromosome; 2008 *kh*, *Kharif* 2008 at New Delhi; 2009 *kh, Kharif* 2009 at New Delhi; 2009-10 *ra*, *Rabi* 2009-10 at Aduthurai

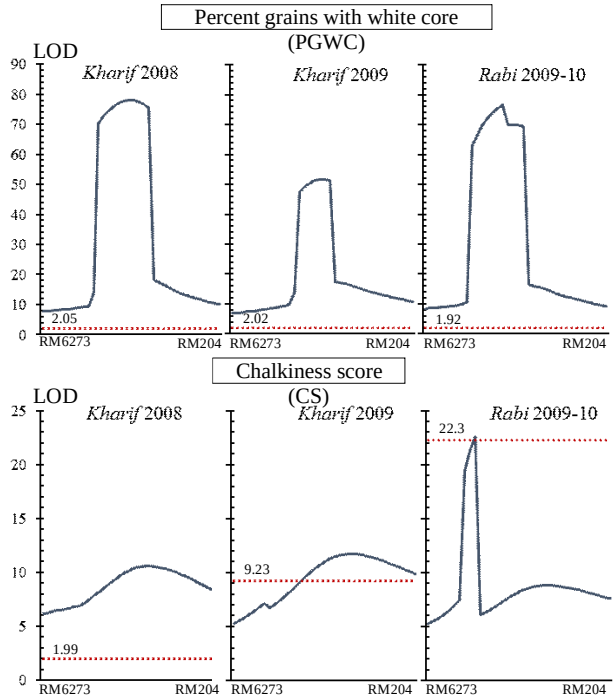

Fig. 2. Peak maps of the major QTL for PGWC and CS mapped on chromosome 6 at marker interval RM6273- RM204 for different seasons. Dotted line indicates LOD threshold

was identified with LOD scores of 78.01, 51.67 and 76.50 respectively for three consecutive seasons and with PVE value of 24.3%, 24.75% and 24.03% respectively. The allele for reduced grain CS was contributed by the parent Jaya. Since this QTL was also found in the same marker interval of *qPGWC6*, and both traits were measuring different degrees of grain chalkiness, and both having same parental allele contribution, there is every reason to assume that both these QTLs refer to same genomic location controlling grain chalkiness.

The QTL for PGWC on chromosome 6 have been reported previously [4, 30-32] but not on the same region as detected in the present study. There are several reports of QTLs affecting grain quality around this region of chromosome 6, primarily because of the presence of waxy (*Wx*) gene that codes for granule bound starch synthase1 (*GBSS1*) enzyme. *GBSS1* is solely responsible for the synthesis of amylose (linear α-1,4-polyglucan) in rice endosperm in non-glutinous rice kernels [33], besides affecting the structure of amylopectin (α-1,6-branched polyglucans) which primarily determines rice eating quality [34, 35]. Mainly there are two waxy alleles reported in rice, *viz*., Wx^a and Wx^b [36], Wx^a allele occurs in *indica*, and Wx^b in *japonica* rices. Wx^a increases amylose content ten times more than that of Wx^b in rice grain [37]. There are two more alleles with limited distribution *viz*., Wx^{in} which shows intermediate behavior of Wx^a and Wx^b, as well as Wx^{op}, which controls opaque or chalky endosperm [38]. Although there are reports relating *Wx* locus and grain chalkiness [39], it is now established that it is not amylose content but alterations of amylopectin structure might be involved in grain chalkiness because severely chalky grains contained amylopectin enriched particularly with long chains compared to slightly chalky grains [40]. Amylopectin, the branched chain starch is synthesized via concerted reactions catalyzed by multiple isoforms of enzymes: soluble starch synthase (SS), starch branching enzyme (BE), and starch debranching enzyme [41]. Further, it is also shown that degree of grain chalkiness is independent of alterations in either of amylopectin chain-length distribution or of amylose content, indicating that different underlying mechanisms may exist for the varietal difference in grain chalkiness [40].

Based on Nipponbare reference genome, the QTL interval, RM6273-RM204 is a large region of 3.03Mbp

Table 4. The quantitative trait loci (QTLs) identified for PGWC and CS evaluated across two location and three seasons by composite interval mapping

QTL	Chr	Marker Interval	LOD	R^2 (%)	TL	Add.	Location	Year
qPGWC6	6	RM6273- RM204	10.6	17.8	2.0	19.3	New Delhi	2008
			11.7	18.8	8.7	16.6	New Delhi	2009
			22.0	12.2	22.6	3.9	Aduthurai	2009-10
qCS6	6	RM6273- RM204	78.0	24.3	2.1	4.0	New Delhi	2008
			51.7	24.8	2.1	3.6	New Delhi	2009
			76.5	24.0	1.9	3.6	Aduthurai	2009-10

LOD, logarithm of odds score; Add., addictive effect; PGWC, percentage of grain with white core; CS, chalkiness score; TL, threshold LOD

at the distal end of the short arm of chromosome 6 that encompasses the *Wx* locus. The marker RM190, which is known to be closely linked to the Wx^b locus [22, 42, 43], lies 1.63Mbp away from RM6273 and 1.40Mbp towards RM204 [13]. The QTL peak identified in this study lying almost in the midway of the marker interval RM6273-RM204 is also suggestive of the presence *Wx* gene as the causal factor of grain chalkiness. So we conclude that, the Wx^b locus from the japonica founders of Pusa1266, could have caused its high grain chalkiness. Additionally, there are reports of association of RM204 with amylose content (AC) and gel consistency (GC) in many germplasm lines [44], although it is located at a genetic distance of 17.7 cM or 1.4 Mbps away from *Wx* locus [45], suggesting that influence on GC might be fashioned by a separate QTL associated with RM204. Nevertheless, this region also contains or lie closer to many other functional alleles such as *alk* locus coding for *soluble starch synthase IIa* (*SSIIa*) controlling gelatinization temperature [46, 47] and other loci governing characters like brown plant hopper resistance (*Bph3)* [42] and also of S_1 locus, a sporo-gametophytic sterility factor [48, 49]. Importance of these loci on grain quality, in *indica-japonica* derived NPTs is a matter of further investigation.

Fine mapping of QTLs *viz., qPGWC6* and *qCS-6,* located in the marker interval RM6273-RM204 and other marker intervals harboring QTLs with minor effect identified in present study will help in resolving genetic control of grain chalkiness leading for their functional validation. Many of the QTL signals with minor effects obtained in this study could be due to larger haplotype blocks in which marker and QTL are farther placed in the absence of intervening markers. Therefore closely placed markers need to be scanned across the RILs to ascertain the validity of such minor QTLs for further exploitation.

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