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Genetic Analysis and Fine Mapping of the *RK4* Gene for Round Kernel in Rice (*Oryza sativa* L.)

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Abstract

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Grain shape of rice is an important trait for both yield and quality. A rice *rk4* (round kernel) mutant was obtained from the *japonica* variety Zhonghua 11 by radiation of ^{60}Co-γ. The grain width of the mutant was increased and the length was decreased. Simultaneously, the 1000-grain weight was slightly reduced, therefore the grain shape of the mutant tended to be small and round. In this study, genetic analysis and gene mapping of the mutant gene were carried out using the F_2 and F_3 populations derived from the mutant and the *indica* variety Xianhui 8006. The results suggested that the round kernel was controlled by a single recessive allele (*rk4*) which was located on chromosome 5. The *RK4* gene was further mapped between the molecular markers LSTS5-77 and LSTS5-60 with 0.57 and 0.096 cM, respectively. A BAC clone was found to span the *RK4* locus, and the *RK4* gene was placed in a 46 kb region that contains six annotated genes according to the available sequence annotation database. This result will help us to isolate the *RK4* gene and reveal the molecular mechanism of the round kernel in rice.

Keywords: BSA; ESEM; kernel shape; SSR; STS

Rice (*Oryza sativa* L.) is one of the most important staple crops that feeds more than one half of the world's population. Grain shape is a key determinant of grain yield by grain length, grain width and grain thickness (Fan *et al.* 2006; Yoon *et al.* 2006; Zuo & Li 2014). Grain shape is recognized as a quantitative trait controlled by multiple genes. Several independent studies identified and functionally characterized numbers of QTLs controlling the rice grain shape. Among these, *GS3* and *qGL3*/*qGL3.1* negatively regulated grain length by encoding a putative transmembrane protein and a protein phosphatase, respectively (Li *et al.* 2004; Fan *et al.* 2006; Mao *et al*. 2010). *GW2,* encoding a RING-type with E3 ubiquitin ligase activity, *qSW5* and *GW5*, encoding a novel nuclear protein of 144 amino acids, negatively regulate grain width (Song *et al.* 2007; Shomura *et al.* 2008; Weng *et al.* 2008). However, *GS5* and *GW8* positively regulated grain width by encoding a putative serine carboxypeptidase and a transcription factor with SBP domain, respectively (Li *et al.* 2011; Wang *et al.* 2012). *TGW6* negatively regulated grain weight and yield by encoding a novel protein with indole-3-acetic acid (IAA)-glucose hydrolase activity (Ishimaru *et al.* 2013). *GL7* and *GW7* positively regulated grain length and improvement of grain quality by encoding a protein homologous to *Arabidopsis thaliana* LONGIFOLIA proteins (Wang *et al.* 2015a, b). The functional characterizations of these genes revealed the molecular mechanisms determining grain shape and weight.

However, additional genes controlling grain shape remain to be identified in rice (Nagata *et al.* 2015; Yin *et al.* 2015; Feng *et al.* 2016), few about the round kernel were detected. To date, 3 genes for the round kernel were reported: *RK1*, controlling short and round grain with slightly flattened shape, was located on chromosome 4; *RK2* and *RK3*, controlling small and round grain, were located on chromosome 10 and 5, respectively (Iwata & Omura 1975, 1984;

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Sanchez & Khush 1998). In this study, we identified a new round kernel gene (*RK4*) controlling the short round kernel using *rk4* mutant, and generated a fine scale map of the genetic region. These results will not only help the future characterization of a molecular mechanism underlying grain size and shape, but also facilitate the design breeding of rice.

MATERIAL AND METHODS

Plant material. The rice round kernel mutant was obtained from a *japonica* variety Zhonghua 11 by radiation of ^{60}Co-γ. After self-pollination over several generations, the mutant was stable and not affected by environmental conditions. The F_2 population for gene mapping was constructed from a cross between the *rk4* mutant plants and *indica* variety Xianhui 8006. To get enough plants for fine mapping the F_3 individuals were developed.

DNA extraction and marker exploration. Total DNA was extracted from fresh leaves of each individual using the cetyltrimethylammonium bromide (CTAB) method with minor modifications (Murray & Thompson 1980). The required density of markers was achieved using published simple sequence repeats (SSR) and sequence tagged sites (STS) (http://www.gramene.org/). PCR was conducted using the standard PCR protocol. The PCR products were separated on a 3.0% agarose gel according to the lengths of the amplified fragments and stained with ethidium bromide.

Molecular mapping of the *RK4* gene. The bulked segregant analysis (BSA) method (Michelmore *et al.* 1991) was performed using two genomic DNA bulks from ten wild-type and ten mutant F_2 plants, respectively. First, SSR markers were employed to detect the polymorphism between the two parents of the segregating population, and the polymorphic markers were further used to detect the polymorphism between two DNA bulks. If a marker was polymorphic between two DNA bulks, it was thought to be putatively linked to the target gene.

Figure 1. Phenotypes for the grain of the wild type and round kernel mutant: comparison with the brown rice (A), comparison with the white rice (B)
bar = 1 cm

Linkage analysis. Linkage analysis was conducted using MAPMAKER/EXP version 3.0b (Lincoln *et al.* 1993). Genetic distances were calculated using the Kosambi mapping function (Lander *et al.* 1987).

Environmental scanning electron microscopy (ESEM) observation. Samples were collected from the middle of the ear at the milky stage. The variances of epidermal cells of glumes between the wild type and *rk4* mutant were observed under a Philips XL 30-ESEM microscope.

RESULTS

Phenotypic characteristics of the *rk4* mutant. During the entire growing period, the mutant plant showed reduced plant height and delayed heading date. And the significant character of the mutant was that its grain shape was rounder than that of the wild type. The grain length, grain width, grain thickness and 1000-grain weight of the mutant and the wild type were also compared. The results showed that the mutant had shorter, wider and thicker grains. At the same time, the grain weight was slightly reduced (Figure 1 and Table 1).

Genetic analysis of the *rk4* mutant. Individual plants of the F_1 and F_2 populations derived from

Table 1. Differences in grain length, width, thickness, and weight between the wild type (WT) and the *rk4* mutant in rice

Trait	WT	F_1 population	*rk4*
Grain length (mm)	8.64 ± 0.57	8.54 ± 0.32	6.50 ± 0.35
Grain width (mm)	3.09 ± 0.19	3.15 ± 0.21	4.03 ± 0.18
Grain thickness (mm)	2.10 ± 0.10	2.19 ± 0.08	2.45 ± 0.13
1000-grain weight (g)	26.60 ± 1.62	26.31 ± 1.35	25.12 ± 0.82

Mean values ± standard deviations

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Table 2. Segregation in grain shape in the F_2 population

Cross	Total	Normal grain	Round grain	Expected ratio	χ^2
rk4 × Xianhui8006	485	366	119	3 : 1	0.056

$\chi^2 0.05 = 3.84$; $df = 1$

the cross between the *rk4* mutant and Xianhui 8006 variety were investigated for the round kernel inheritance. All F_1 plants exhibited a wild type phenotype, and the ratio of wild type to mutant was 3 : 1 in the F_2 population (Table 2 and Figure 2). It is suggested that the round kernel trait was controlled by a recessive allele of single gene.

Environmental scanning electron microscopy (ESEM) observation. Through observing the epidermal cells of glumes by ESEM, we found that the cells of the mutant were wider and shorter compared with the wild type. The result showed that the epidermal cells of the *rk4* mutant had increased width and decreased length (Figure 3). As a result, the mutant grain was rounder than that of the wild type.

Mapping of the *RK4* gene. The polymorphisms were examined with about 600 pairs of SSR and STS primers which were evenly distributed on 12 chromosomes, and 100 of them exhibited polymorphisms between the round kernel mutant and Xianhui 8006 variety. Six pairs of primers (RM6317, RM17962, RM18055, S5-1, S5-18, S5-21) revealed polymorphisms between the two DNA bulks. The 6 pairs of primers were further employed to preliminarily map the *RK4* gene with the F_2 population. The *RK4* gene was located between S5-18 and RM17962. Then, 521 recessive homozygotes were identified from a large F_3 population and used for fine mapping. Seventy-seven pairs of STS primers were synthesized on the genome sequence between S5-18 and RM17962. Twenty-seven pairs of primers were found to be polymorphic between the two bulks. By genetic linkage analysis, the *RK4* gene was located between the molecular markers LSTS5-77 and LSTS5-60, at distances of 0.57cM and 0.096cM, respectively

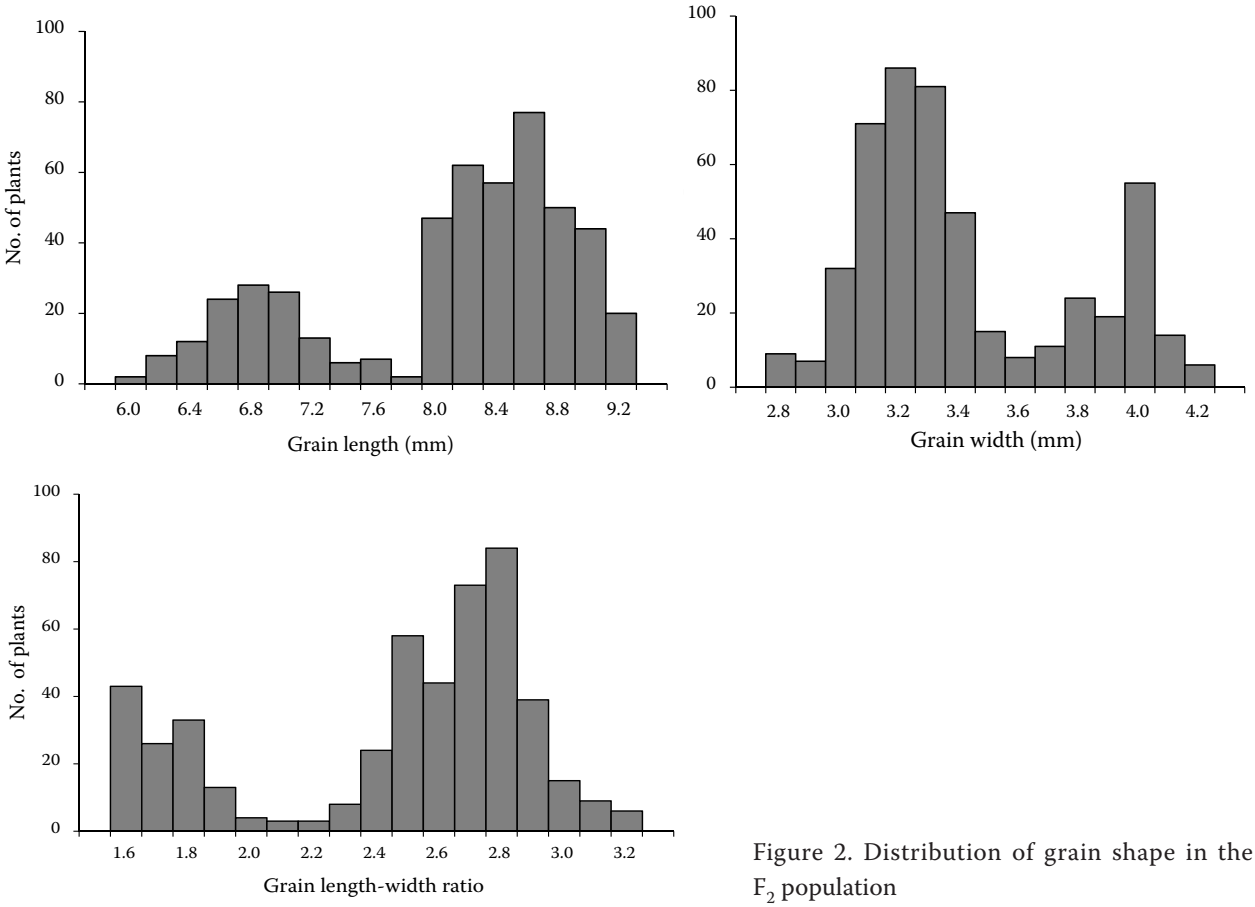

Figure 2. Distribution of grain shape in the F_2 population

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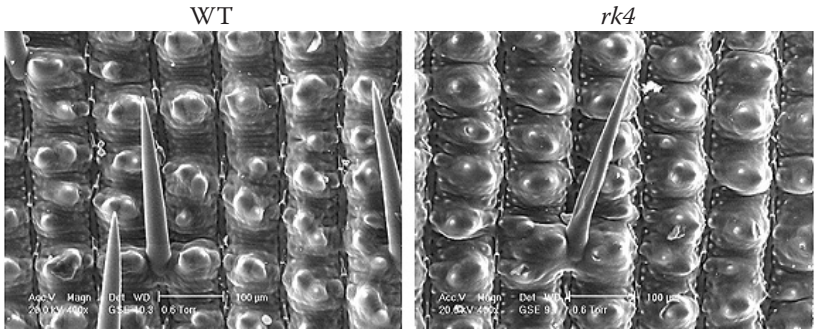

Figure 3. Environmental scanning electron microscope (ESEM) comparison of the grain between the wild type and the *rk4* mutant (scale bar: 100μm); the width and length of the epidermal cells in *rk4* mutant were 123.4 ± 2.7 μm and 97 ± 1.58 μm, respectively, and in the wild type 104.1 ± 1.45 μm and 112.8 ± 1.99 μm (mean values ± standard deviation)

Table 3. The primers used in fine mapping of the *RK4* gene

Marker	Forward primers	Reverse primers
LSTS5-14	CGACAAGATTGGGTGAGT	TGAAAGCGAGAAAGGTTC
LSTS5-29	TGGCGATGAATTGGTAAG	ATTTGATTTGAAAGGAGGC
LSTS5-60	AGGGGAATCAATGCTGT	GAAGGATTCTGTTTTGTTGA
LSTS5-77	ATTGTTTGCCTTGGTTGT	CCTTATCTCCCAGGTTGC

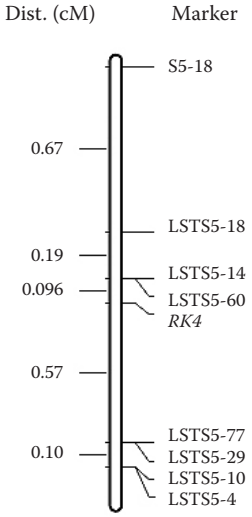

Figure 4. The fine mapping of the *RK4* gene on chromosome 5

(Table 3, Figure 4). Moreover, these two markers were located in the BAC clone P0676G05 according to the Nipponbare genome (http://www.gramene.org) with physical distance of 46 kb (Figure 5).

Candidate genes in the 46 kb region. There are six annotated genes in the 46 kb region according to the available sequence annotation database (http://ricegaas.dna.affrc.go.jp/). LOC_Os05g6510 encodes a protein containing a 4Fe-4S binding domain and has a corresponding full-length cDNA (AK109316). LOC_Os05g6520 encodes a protein containing helix-loop-helix DNA-binding domain and has a corresponding EST (EA702257). LOC_Os05g6530 and LOC_Os05g6540 are both the expressed proteins and have the corresponding EST (CB655890) and full-length cDNA (AK101218), respectively. LOC_Os05g6550 and LOC_Os05g6560 are both the hypothetical proteins.

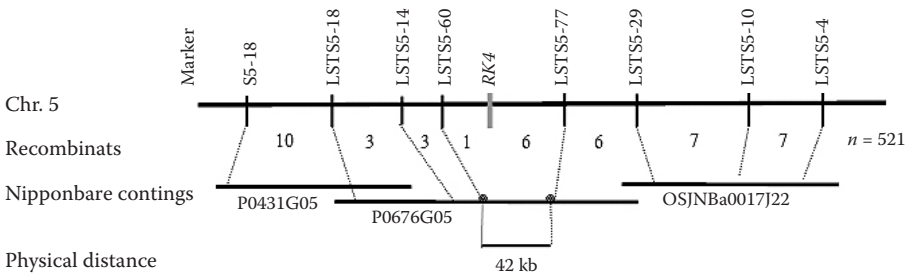

Figure 5. The BAC contigs encompassing the *RK4* gene

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DISCUSSION

The map-based cloning approach is a method to isolate the interested gene based on an intensive genetic and physical mapping. In this study, the round kernel trait was controlled by a single recessive allele. The gene was named *RK4* after *RK1*, *RK2* and *RK3*, and was placed between the markers LSTS5-77 and LSTS5-60 in the BAC clone P0676G05. There are six candidate genes to predict the *RK4* in the 46 kb region according to the annotation system of rice. The results will be helpful for us to isolate the candidate gene successfully in the following study.

In rice, more than 400 QTLs that control grain shape traits have been detected and at least 20 genes have been isolated by map-based cloning strategies (Huang *et al.* 2013; Zuo & Li 2014; Hu *et al.* 2015; Liu *et al.* 2015a, b; Wang *et al.* 2015a, b). Compared with the results of previous studies, it can be deduced that *RK4* should not be the same locus as the report by Tan *et al.* (2000) detecting a QTL between the RFLP markers RG360 and C734a affecting grain width and length-width ratio, and it is not either as the report by Xing *et al* (2002) detecting a QTL between the RFLP markers R3166 and RG360 controlling grain weight. Moreover, *RK4* is not the allele of *GW5* or *qGW-5* (Wan *et al.* 2008; Wang *et al.* 2008) which is on the BAC clone OJ1097_A12, but a new gene locus controlling the round kernel on the short arm of chromosome 5.

The molecular and cellular mechanisms of seed development and seed size were described in the past few years. Anatomically and genetically, the embryo, the endosperm and the testa consist of the different genetic composition of the seed. It was obviously the seed growth and development that were controlled by the interactions among the three seed components. In *Arabidopsis thaliana*, *IKU1*, *IKU2* and *MINI3* controlled seed size in the same pathway by reduced growth and early cellularization of the endosperm (Alonso-Blanco *et al*. 1999; Luo *et al*. 2005). In rice, the *GIF1* which increased grain weight and production encoded a cell-wall invertase required for carbon partitioning during early grain-filling (Wang *et al.* 2008). The *SLG7*, encoding a protein homologous to *A. thaliana* LONGIFOLIA1 and LONGIFOLIA2, produced long and thin grains by longitudinally increasing cell length while transversely decreasing cell width, which is independent of the cell division (Zhou *et al.* 2015). In our work, it was identified that the width of the epidermal cells of glumes increased and the length decreased for the *rk4* mutant. As a result, in comparison with the wild type, the grain shape of the *rk4* mutant appeared smaller and rounder and the grain weight decreased slightly. It is believed that the cloning and functional analysis of the *RK4* gene will help us to reveal the molecular mechanism of the round kernel in the future.

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