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

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Abstract

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 60Co-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 F2 and F3 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;
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 60Co-γ. After self-pollination over several generations, the mutant was stable and not affected by environmental conditions. The F2 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 F3 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 F2 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.

**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 F1 and F2 populations derived from

<table>
<thead>
<tr>
<th>Trait</th>
<th>WT</th>
<th>F1 population</th>
<th>rk4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grain length (mm)</td>
<td>8.64 ± 0.57</td>
<td>8.54 ± 0.32</td>
<td>6.50 ± 0.35</td>
</tr>
<tr>
<td>Grain width (mm)</td>
<td>3.09 ± 0.19</td>
<td>3.15 ± 0.21</td>
<td>4.03 ± 0.18</td>
</tr>
<tr>
<td>Grain thickness (mm)</td>
<td>2.10 ± 0.10</td>
<td>2.19 ± 0.08</td>
<td>2.45 ± 0.13</td>
</tr>
<tr>
<td>1000-grain weight (g)</td>
<td>26.60 ± 1.62</td>
<td>26.31 ± 1.35</td>
<td>25.12 ± 0.82</td>
</tr>
</tbody>
</table>

Mean values ± standard deviations
the cross between the \( r k4 \) 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 \( r k4 \) 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 0.57 cM and 0.096 cM, respectively.
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.

![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).](image)

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

<table>
<thead>
<tr>
<th>Marker</th>
<th>Forward primers</th>
<th>Reverse primers</th>
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<tbody>
<tr>
<td>LSTS5-14</td>
<td>CGACAAGATGCGGGATGAGT</td>
<td>TGAAACGCAGAAAAAGGTTTC</td>
</tr>
<tr>
<td>LSTS5-29</td>
<td>TGGCGATGAATTGGTAAAG</td>
<td>ATTTGATTGGAAAGGAGGC</td>
</tr>
<tr>
<td>LSTS5-60</td>
<td>AGGGGAAATCAATGCTGT</td>
<td>GAAGGATTCTGTTTGGTTGA</td>
</tr>
<tr>
<td>LSTS5-77</td>
<td>ATTGTGTCCCTTTGGTTGT</td>
<td>CCTTATCTCCCAGGTTGC</td>
</tr>
</tbody>
</table>

![The fine mapping of the RK4 gene on chromosome 5](image)

![The BAC contigs encompassing the RK4 gene](image)
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 GWS or qGW-5 (Wang 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, IKU11, IKU12 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 longitudinal 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.

References


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