RL3(t), Responsible for Leaf Shape Formation, Delimited to a 46-kb DNA Fragment in Rice

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Abstract: Two mutants with rolled leaves, temporally designated as rl3(t)-1 and rl3(t)-2, were served for exploring the mechanism underlying the rolled leaf characteristic. Except for having typical rolled leaves, the plant heights and panicle lengths of rl3(t)-1 and rl3(t)-2 significantly decreased, and the seed-setting rate also decreased when compared with wild type 93-11. Cytological analysis suggested that the rolled leaf phenotype might be caused by the changes of number and size of bulliform cells. Genetic analysis indicated rl3(t)-1 is allelic to rl3(t)-2, and controlled by a recessive gene. Gene mapping result indicated that RL3(t) gene resided in a 46-kb long region governed by the sequence tag site markers S3-39 and S3-36 on rice chromosome 3. The result provides an important clue for further cloning the RL3(t) and understanding the mechanism of rice leaf development.

Key words: gene mapping; leaf shape formation; mutant; rice; rolled leaf gene

Leaf is the main organ for photosynthesis, and its morphology is closely related to the photosynthesis efficiency and consequently contributing to the yield formation. Recently, Yuan and other breeders proposed that combination of heterosis utilization with plant ideotype is the only way to achieve higher yield (Chen et al, 1989; Yuan, 1997). The morphology of rice leaf is one of the major components of the ideotype in rice. And being appropriate curled can be useful for maintaining the rice leaf upright and helping the population to receive more light (Lv et al, 1991; Su et al, 2003; Kang et al, 2007).

Because of the importance of leaf shape and size in constructing ideotype in rice, many scientists have put efforts in dissecting the genetic mechanism underlying rice leaf formation by analyzing the leaf shape mutants (Zuo and Li, 2014). To date, a few genes involved in regulating the development of leaf shape, especially the curled leaf shape, have been cloned and functionally analyzed. SLL1/RL9, a member of the KANADI family, encodes a transcription factor (Yan et al, 2008; Zhang et al, 2009), and its mutation leads to the failure of programmed cell death of abaxial mesophyll cells and the suppression of the differentiation of the abaxial cells, and finally to generate adaxially rolled leaves. ROC5 gene encodes a protein containing a leucine zipper domain, homologous to GLABRA2 in Arabidopsis, and is proven to negatively regulate the development of the bulliform cells. The number and size of the bulliform cells increased when ROC5 gene was knocked out, consequently leading to generate adaxially rolled leaves (Zou et al, 2011). ACL1 gene encodes a protein with unknown function. The expression of acl1 in mutant is higher than that in wild type. Increasing number and size of bulliform cells in mutant is the cause for producing rolled leaves (Li et al, 2010). In addition, SRL1, encoding a putative glycosylphosphatidylinositol-anchored protein, negatively regulates the formation of bulliform cells by suppressing the expression of H+-ATPase subunits and H+-pyrophosphatase genes (Xiang et al, 2012). In srl1 mutant, there are more bulliform cells at the adaxial layers, which lead to produce adaxially rolled leaves. ADL1 encodes a calpain-like cysteine proteinase, and its mutation leads to the change of expression pattern of HD-ZIPIII (Class III homeodomain-leucine zipper) in mature leaves, which usually expresses in adaxial side of leaves and is a key factor in determining leaf abaxial layer. However, in adl1 mutant, the bulliform cells originally exist in abaxial side was found to grow all over the leaves, consequently causing the adaxially rolled leaves (Hibara et al, 2009). The ND1 gene encodes a cellulose synthase-like protein D4 (Li et al, 2009). The cell walls, full of high electron density materials in parenchymatous tissue, were found to thicken abnormally in nd1 mutant, leading to produce narrow and rolled leaves. RL14 gene is shown to encode a 2OG-Fe (II) oxygenase protein. The secondary cell wall is negatively regulated in rl14 mutant, and thus water transport is affected. As a result, bulliform cells become shrink as well as stomatal complexes grow smaller, and finally leads to incurved leaves (Fang et al, 2012). Although many genes have been cloned and detailed characterized, the
relationship between these cloned genes involved in rice leaf formation is still not very clear yet. In order to elucidate molecular mechanism regulating the development of leaves, more information of genes responsible for leaf development is needed in characterizing rice leaf shape mutants (Gao and Lv, 2006; Yan et al, 2008; Chen et al, 2010).

In the present study, two mutants with rolled leaves, derived from the indica variety 93-11 via the radiation of \(^{60}\text{Co-\gamma}\) ray in M\(_2\) generation, were served as materials for morphological analysis and gene mapping. The result provides an important clue for further cloning the \(RL3(t)\) and understanding the mechanism of rice leaf development.

**MATERIALS AND METHODS**

**Rice materials**

Two mutants with rolled leaves, derived from the indica variety 93-11 via the radiation of \(^{60}\text{Co-\gamma}\) ray in M\(_2\) generation, were temporally designated as \(rl3(t)\)-1 and \(rl3(t)\)-2, and this character can inherit stably across generations. The wild type (WT) indica 93-11 and japonica Wuyunjing 8 (WYJ8) were served as materials for genetic analysis and gene mapping.

**Morphological analysis**

The morphology of gross plants and leaves of wild type and \(rl3(t)\)-1 were observed at 50 d after seeding. For each sample, five plants were randomly selected for traits measurement, including plant height, panicle length, number of grains per panicle and seed-setting rate. For cytological analysis, the fresh leaves were harvested from 20-day seedlings of 93-11 and \(rl3(t)\)-1 grown in the greenhouse, and hand-section was performed to observe and photo under light microscope and fluorescence microscope. At the same time, the numbers of large and small vascular bundles were counted with three replicates.

**Genetic analysis and fine-mapping of \(RL3(t)\)**

The two mutants \(rl3(t)\)-1 and \(rl3(t)\)-2 were crossed to the wild type 93-11 and the japonica variety WYJ8, respectively. Four F\(_2\) populations were generated for analyzing the genetic property of \(RL3(t)\), and the two of them were also employed as mapping populations. Furthermore, a cross between the two mutants was also made to test whether they are allelic or not.

For roughly mapping the \(RL3(t)\) gene, ten plants with normal leaves and ten plants with rolled leaves were firstly selected from the F\(_2\) \((rl3(t)-1/WYJ8)\) segregation population to construct two DNA pools according to the BSA (bulk segregant analysis) strategy. SSR markers showing polymorphic between two parents were used to detect the polymorphism between the two DNA pools. The polymorphic markers between the two DNA pools were considered as putative linked markers.

An enlarged F\(_3\) population derived from the plants with normal leaves in F\(_2\) population \((rl3(t)-1/WYJ8)\) was generated, and the incurred individuals were collected as segregation population for fine-mapping of \(RL3(t)\). Meanwhile, more SSR markers, new developed STS markers, and CAPs (cleaved amplified polymorphism) markers around the \(RL3(t)\)-anchored region were used. The primer sequences of SSR markers were downloaded from the web site (http://www.gramene.org). The new STS and CAPs markers were developed according to the diversity between the genomic sequences of Nipponbare and 93-11 around the target regions. And the primer sequences of STS and CAPs were designed using Primer Premier 5.0 software and synthesized by Shanghai Sangon Inc.

DNA was extracted from fresh-frozen leaves of each plant using the CTAB (cetyl trimethyl ammonium bromide) method. DNA amplification was performed, programmed for an initial 5 min at 94 °C, then followed by 32 cycles for 1 min at 94 °C, 1 min at 55 °C, 1.5 min at 72 °C, and finally 10 min at 72 °C. A volume of 20 µL reaction was designed, containing 10 mmol/L Tris-HCl (pH 8.3), 50 mmol/L KCl, 1.5 mmol/L MgCl\(_2\), 1 U Taq polymerase, 4 mmol/L dNTP, 10 pmol/L primers, 20 ng DNA template. The PCR products were directly analyzed on 3% agarose gels stained with ethidium bromide, and photographed with a GEL DOC 1000 system.

**RESULTS**

**Characterization of \(rl3(t)\) mutant**

Compared to the wild type, the two mutant \(rl3(t)\)-1 and \(rl3(t)\)-2 exhibited typically incurved leaves. Moreover, other agronomic traits were also found to change significantly (Fig. 1-A, B, C). The plant height of two mutants are much shorter than that of WT, significantly decreased with about 20 cm (Fig. 1-C, E), and the panicle length of the two mutants is 84% of that of WT (Fig. 1-D, F). In addition, the grain numbers per panicle of mutants are about 18%-20% lower than that of WT (Fig. 1-G), and the two mutants exhibited significantly lower seed-setting rate (66% and 55%, respectively) (Fig. 1-H). Besides the traits mentioned above, there was no significant changes between the two mutants and WT. Therefore, \(RL3(t)\) gene not only plays an important role in the development of leaf, but also affects plant height and panicle developments.

**Cytological analysis of leaf**

In order to find the reason why the leaves of mutants are rolled, hand-section was performed in the mature leaves of both the WT and mutant \(rl3(t)\)-1 at the seeding stage. The result showed that the number of vascular bundles of leaves in \(rl3(t)\)-1 significantly decreased when compared with that in 93-11 (Fig. 2). The numbers of large and small vascular bundles in WT were 14.3 and 49.3, respectively, while those in \(rl3(t)\)-1 mutant were 8.3 and 26.3, respectively. It was found that there was no significant difference in terms of morphology of both large and small vascular bundles. However, there were more and well-distributed bulliform cells in the WT than in the mutant. In
addition, the volume of the bulliform cells in the middle part of rl3(t)-1 mutant were bigger than those of the WT (Fig. 3). The result indicated that the change of the morphology of the bulliform cells may be the reason of the adaxial rolled leaves.

Genetic analysis of rl3(t)-1 and rl3(t)-2 mutants

Crossing between the two mutants, the F1 (rl3(t)-1/rl3(t)-2) plants exhibit rolled leaf, suggesting they are allelic. Two F2 segregation populations were generated by crossing two mutants with a japonica cultivar WYJ8 and its wild type 93-11, respectively. The F1s have normal leaves, and in the F2 population, the segregation of leaf shape was clearly observed. However, χ²-test showed the segregation for leaf shape was significantly distorted from 3:1 Mendel’s ratio, with severely fewer adaxially rolled leaf plants (Table 1).

Molecular mapping of RL3(t) gene

Initially, two DNA pools were constructed with ten plants with normal and rolled leaves, respectively, from the F2 population (rl3(t)-1/WYJ8 and rl3(t)-2/WYJ8), subsequently for polymorphic marker detection. It was found that SSR marker RM6676 on chromosome 3 exhibited polymorphic between two DNA pools. Based on the result, 46 mutant individuals from F2 segregation population were selected to constitute the mapping population, and genotyped with more SSR markers and STS markers around RM6676. Linkage analysis showed that RL3(t) gene was located in the region between SSR marker RM273 and STS marker M3-45, with the genetic distances of 5.5 cM and 4.4 cM, respectively, on the long arm of chromosome 3 (Fig. 4-A).

For fine mapping the RL3(t) gene, an enlarged F3 population (the progeny of plants with normal leaf in F2 population) was generated, and 430 individuals with adaxially rolled leaves were obtained for fine mapping of RL3(t). Meanwhile, more STS and CAPs markers were newly developed in the region between RM6776 and M3-45, of which, six STS markers showed polymorphic between the mutants and WYJ8 (Table 2). Using these markers, we genotyped 430 recessive plants, consequently, three individuals had no recombinant events at

<table>
<thead>
<tr>
<th>Cross</th>
<th>F1 Normal</th>
<th>F2 Mutation</th>
<th>Total</th>
<th>χ² (3:1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>93-11/rl3(t)-1</td>
<td>1538</td>
<td>312</td>
<td>1850</td>
<td>67.93**</td>
</tr>
<tr>
<td>Wuyunjing 8/rl3(t)</td>
<td>499</td>
<td>46</td>
<td>540</td>
<td>86.34**</td>
</tr>
<tr>
<td>rl3(t)-2/93-11</td>
<td>768</td>
<td>114</td>
<td>882</td>
<td>64.86**</td>
</tr>
<tr>
<td>rl3(t)-2/Wuyunjing 8</td>
<td>682</td>
<td>78</td>
<td>760</td>
<td>87.24**</td>
</tr>
</tbody>
</table>

**Significance at the 0.01 level.
the three STS markers: M3-28, M3-13 and S3-39, while exhibited single crossing-over at the other three markers, including S3-36, S3-41 and M3-16. On contrast, another plant was found to have a single crossing-over at the former three markers loci, but no crossing-over at the later three markers. Similarly, one individual displayed double crossing-over at the former three markers and no crossing-over at the later three markers. In addition, one plant was found to exhibit no crossing-over at the marker S3-36 locus, but had double crossing-over at the other five markers. Similarly, one individual has the same WT genotype at marker S3-39 with single crossing-over at the other five markers (Fig. 4-B). On the basis of the genotypic information of recombinants, RL3(t) gene was successfully assigned to a region governed by S3-36 and S3-39, with about 46-kb long DNA fragment (Fig. 4-B).

**DISCUSSION**

The morphology of rice leaf is one of the major components of the ideotype in rice, which is a target to improve in rice breeding program. Being appropriate curved leaves are useful for promoting both photosynthetic efficiency and planting density. In the present study, two mutants with rolled leaves, derived from the indica variety 93-11, were subjected to genetic analysis and gene mapping. RL3(t) gene was finally delimited to a 46-kb long DNA region governed by the STS markers S3-39 and S3-36. In the target region, there are eight open reading frames (ORFs) (http://rice.plantbiology.msu.edu/). It has been found that two ORFs were predicted to encode proteins containing the F-box domain, and two encode nucleo-proteins. In addition, one zinc finger protein gene, and one amylase gene as well as one DVR gene are also been predicted. Furthermore, an ORF was predicted to encode an unknown function protein. Previously, there were two genes, NRL2(t) (Wang et al, 2011) and OsAGO7 (Shi et al, 2007), locating on rice chromosome 3, which were reported to be responsible for the development of leaf shape. Based on their location information, it was found that NRL2(t) and OsAGO7 are not in the RL3(t) anchored region, indicating that RL3(t) gene is a novel gene, not reported yet.

Leaf is the main organ for photosynthesis and transpiration. Its development consists of the initiation of leaf primordium and establishment of leaf polarity. The accumulated knowledge indicates that many factors are involved in the process of leaf development, including transcription factors, small RNAs and auxin (Yan et al, 2008; Zou and Li, 2013), but the genetic mechanism of rice leaf development is still unclear yet. Much more mutants, such as narrow leaf mutants, rolled leaf mutants, are needed for analyzing and cloning the genes which may play important roles in leaf development. If so, we are able to provide more information on the regulation network in the process of leaf shape formation, and thus, providing an important clue for improving the morphology of leaves through genetic engineering, and laying a solid foundation for constructing the ideotype of rice. In present study, two rolled leaf mutants rl3(t)-1 and rl3(t)-2 were obtained, and the gene mapping was successfully performed using molecular markers. The knowledge of rice leaf development would be enriched as the RL3(t) being cloned and functionally analyzed.

It should be noted that, in the process of the analysis of genetic mode of RL3(t), the segregation for leaf shape is significantly distorted from 3:1 Mendel’s ratio in the F2 population, especially in the F2 population [rl3(t)-2/WYJ8], and the number of rolled leaf individuals is much less than expected. There are two possible reasons causing this phenomenon, one reason is that, the typical indica and japonica germplasm served as materials for construction of genetic population may have enough polymorphic molecular markers between them, which will facilitate gene mapping. However, indica and japonica belong to two subspecies of Asian cultivar. Thus, crossing between them usually exhibits semi-sterile property due to their relatively far phylogenetic relationship, causing the segregation distortion in the progeny. Matsushita et al (2003) proposed that the distorted segregation markers usually cluster in linkage groups due to gametophytic selection and zygotic selection. In general, gametophytic selection plays a predominant role in segregation distortion in rice (Xu et al, 1997). In previous studies, there were 11 gametophytic genes identified to be responsible for

**Table 2. New-developed molecular markers in present study.**

<table>
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<tr>
<th>Marker</th>
<th>Primer sequence (5′-3′)</th>
<th>Location</th>
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<tbody>
<tr>
<td>M3-28</td>
<td>F: TATCACCAGAGCATAAAACAAAC&lt;br&gt;R: GAATGAGCTCTCTACCCAAATGGAGGAAAT</td>
<td>AC137547</td>
</tr>
<tr>
<td>M3-13</td>
<td>F: TATCAAGGGGGGAAAGCC&lt;br&gt;R: ACATCCGACAAAGACAGG</td>
<td>AC121491</td>
</tr>
<tr>
<td>M3-16</td>
<td>F: AGGTGACTTTCCTCTTGC&lt;br&gt;R: TACATTGGGTATGTTGT</td>
<td>AC136284</td>
</tr>
<tr>
<td>S3-39</td>
<td>F: AGCAGAGGCTCCATCAC&lt;br&gt;R: TCTTCTTTCACCCCTCAT</td>
<td>AC135257</td>
</tr>
<tr>
<td>S3-36</td>
<td>F: CTCAGTGGTCTGTACGA&lt;br&gt;R: CGGATGATGATGGAAAT</td>
<td>AC135257</td>
</tr>
<tr>
<td>S3-41</td>
<td>F: ACCGAGCAGCTGAGAAG&lt;br&gt;R: AAGAGGGGAAGCCAGAT</td>
<td>AC136284</td>
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constructed a rice genetic linkage map comprising 92 SSR
markers, and found 9 segregation distortion regions (SDR),
including SDR1-1, SDR1-2, SDR2, SDR3-1, SDR3-2, SDR6,
SDR8, SDR11 and SDR12. The RL3(t) gene is in the region of
SDR3-1, which causes the segregation distortion for leaf shape.
Another reason is that individuals with some kinds of genotype
are less competitive. Compared with WT, the individuals with
rolled leaves usually exhibit weak in growth and lower germinating
rate, possibly resulting in the decrease number of plants with
rolled leaves in F2 population. In the F2 population
(93-11/rl3(t)-1 and rl3(t)-2/93-11), the ratios of the number of
plants with normal leaves to those with rolled leaves are 5:1
and 7:1, respectively, which are lower than the ratio of 15:1, the
two genes segregation mode in F2 population. Taken together, it
was deduced that rl3(t) mutant is controlled by a recessive gene.

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