Characteristics and Genetic Segregation of a Rolled Leaf Mutant in Rice

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Abstract: Leaf structure is one of the important agronomic traits. A rolled leaf mutant was induced from an ethyl methane sulfonate (EMS)-treated japonica rice, ‘Koshihikari’. The rolled leaf mutant showed phenotypes of reduced leaf width and leaf rolling. In addition, several abnormal morphological characteristics were observed, including dwarfism, defected panicle, delayed germination, and lower seed-setting. Microscopic analysis revealed that the number of small veins was decreased and the sizes of adaxial bulliform cells were reduced in the mutant leaves. The genetic study with two F2 populations from the crosses of the rolled leaf mutant with ‘Koshihikari’ and Milyang23 suggested that the mutant phenotype might be controlled by a single dominant gene.

Key words: Rice (Oryza sativa L.), Rolled leaf, Genetic analysis

INTRODUCTION

Plant leaf is the main photosynthetic organ and the shape of leaf is important in light capture, carbon fixation, and gas exchange for photosynthesis (Govaerts, 1996). Moderate leaf rolling contributes to the erectness of leaves, which can increase the light transmission rate (Duncan, 1971). Thus, the study of rolled leaf mutant will be meaningful for breeding crops with improved photosynthetic efficiency and subsequently higher yield (Lang et al., 2004). Leaf rolling phenotypes are regulated by complicated developmental processes, including pattern formation, polarity establishment, and cell differentiation (Micol et al., 2003; Lang et al., 2004). Osmotic pressure affects leaf rolling between internal and external tissues, as well (Hsia et al., 1984). However, the mechanism underlying leaf rolling in monocots remains to be elucidated.

In rice, many studies for the genes controlling leaf rolling have been reported. Up to the present, 11 rolled leaf (rl) mutants in rice have been characterized. Among them, six recessive genes of narrow or rolling leaf rl1, rl2, rl3, rl4, rl5, and rl6 were mapped on the rice chromosomes 1, 2, 12, 1, 3, and 7 by morphological markers, respectively (http://www.gramene.org/). A dominant gene controlling the leaf rolling was reported by Liu et al. (1998). The QTLs controlling the leaf rolling were mapped by molecular markers. rl7 and rl8 located at different regions on chromosome 5 and rl9(t) is located on chromosome 9 (Yan et al., 2005). The incomplete recessive rolled leaf mutant, rl9(t), was fine-mapped to a 137-kb region on chromosome 2 (Shao et al., 2005).

Much less is known about the molecular mechanism that control leaf shape in rice. Recently, several genes for leaf development were identified in rice. ROLLED LEAF (RL9) / SHALLOT-LIKE1 (SLL1) encodes a KANADI family protein and is involved in leaf abaxial cell development (Yan et al., 2008; Zhang et al., 2009). The environmental effects such as water stress was also involved in leaf rolling. CONSTITUTIVELY WILTED1 (COW1) / NARROW LEAF7 (NAL7) encodes a YUCCA protein family. Mutation in NAL7 resulted in reduced leaf width and leaf rolling (Woo et al., 2007; Fujino et al., 2008). NARROW LEAF1 (NAL1) is a plant-specific protein with unknown biochemical function. NAL1 mutant shows narrow leaf phenotype because of decreasing number of longitudinal veins (Qi et al., 2008). ADAXIALIZED LEAF1 (ADL1) encodes a calpain-like cysteine protease that is associated with the maintenance of axis formation in
leaf development (Hibara et al., 2009). Rice OsAFO7 gene encodes an argonaute (AGO) family protein. And the overexpression of OsAFO7 resulted in the upward curling of rice leaves (Shi et al., 2007). In the recent study, a narrow and rolled leaves mutant, narrow and rolled leaves 1 (NRL1), was mapped to the chromosome 12. The NRL1 gene encodes the cellulose synthase-like protein D4 (OsCslD4) that affected morphogenesis and vegetative development in rice (Jiang Hu et al., 2010).

Here, we characterized the rolled leaf mutant which was induced by ethyl methane sulfonate space (EMS) and investigated the inheritance of this mutant phenotype.

**MATERIALS AND METHODS**

**Plant materials**

A rolled leaf mutant was induced by the chemical mutagen EMS from a japonica cultivar, Koshihikari, in the Crop Molecular Breeding Laboratory of Department of Plant Science, Seoul National University (SNU). The rolled leaf mutant was crossed with Koshihikari and Milyang 23 respectively, to produce the F2 population which was used for genetic analysis. All rice plants were grown in an experimental field at the SNU in Suwon, Korea during the natural growing season. The conventional cultivation method by Crop Molecular Breeding Lab was applied.

**Pollen fertility assessment**

Spikelets were harvested just before flowering, and then fixed in 3:1 ethanol : acetic acid (v/v) fluid for 1 hour. Pollens were placed on slides with 1% (w/v) iodine potassium iodide (I₂-KI) and 1% (w/v) acetocarmine solution, respectively (Chaudhary et al. 1981). The pollen fertility was examined under an optical microscope.

**Leaf sections and microscopic analysis**

Paraffin-embedded leaf tissue sections were prepared by the methods by Ji et al. (2006) with slight modifications. Leaves were harvested at the early stage and fixed in FAA solution (50% ethanol, 5% acetic acid, 3.7% formaldehyde). The tissue samples were dehydrated in graded ethanol series to 100%, embedded in paraffin, cut ~20 μm sections using a microtome (HM340E, MICROM Lab), and adhered to glass slides. The sections were deparaffinized using xylene, rehydrated using a graded ethanol series, before staining with Safranin O (Sigma-Aldrich, St Louis, MO). Finally, cross sections of leaf were observed by optical microcopy at 50 and 100 x magnifications.

**Genetic analysis of rolled leaf mutant**

In summer of 2009, the rolled leaf mutant was crossed to the parental variety Koshihikari and Milyang 23 (tongil-type). The F1 seeds were planted in the green house in the same year. In the spring of 2010, two F2 populations derived from the cross between the mutant and two varieties: ‘Kosihikari’, Milyang23, respectively. A F2 population from the cross between the mutant and Koshihikari was used for genetic analysis of the rolled leaf, and the other F2 population from the cross between the rolled leaf mutant and Milyang23 was used for BSA (bulked segregant analysis). For BSA test, DNA bulks were made from 10 F2 plants showing rolled leaf phenotype and 10 normal F2 plants, respectively. A total of 106 STS markers of known chromosomal position covering all chromosomes evenly were used in BSA test. The 106 STS markers were designed in Crop Molecular Breeding Laboratory (Chin et al., 2000).

**RESULTS & DISCUSSION**

The morphology of the mutant was compared with that of the Koshihikari. The mutant leaves were narrow and rolled upwardly. From the seedling stage, mutant plants can be distinguished from the Koshihikari by narrow and rolled leaf phenotypes. Apart from narrow and rolled leaves, other defects in the mutant were also observed. When compared with the Koshihikari, the plant height was significantly changed. At the seed setting stage, the mutant was determined to be shorter than Koshihikari (28.2 ± 3.5 cm compared with 91.3 ± 2.5 cm in the wild type; n=30, P<0.001) (Fig. 1A, B), which was approximately 31% of the height compared
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Fig. 1. Phenotypes of wild-type and rolled leaf mutant
A. Plants of wild-type (left) and rolled leaf mutant (right)
B. Plants of wild-type (left) and rolled leaf mutant (right) after heading
C. Comparison of panicle length and length of upper six internodes (I, II, III, IV, V or VI) among wild-types (left) and rolled leaf mutants (right)
D. Abnormal root development of the wild-types (left) and mutants (right)
E. Traits of wild-types and rolled leaf mutants

Fig. 2. Panicle and floral organ phenotypes of wild-type and rolled leaf mutant
A. Panicle of wild-type (left) and mutant (right)
B. Spikelets of wild-type (left) and rolled leaf mutant (right)
C. Floret organ of wild-types (left) and rolled leaf mutants (right)
D. Stamens and carpel of wild-type (left) and mutant (right)
E. Evaluation of pollen fertility by staining with acetocarmine between wild-type (left) and mutant (right)
F. Evaluation of pollen fertility by staining with I$_2$-KI between wild-type (left) and mutant (right)
G. Altered seed development of wild-type (left) and rolled leaf mutant (right)

The Koshihikari Plant height of the mutant was reduced mainly due to the shortened the second and the third internode elongation. Takeda (1977) categorized rice dwarf mutants into six groups according to elongation patterns of the upper five internodes. In d6-type mutant, the second and the third internodes below the uppermost are shortened. Thus, the mutant could be grouped into d6-type according to the definition by Takeda (Fig. 1C). In roots, the mutant showed defected growth. It has shortened primary roots (43.8% reduction) (Fig. 1D) and the numbers of lateral and adventitious roots were reduced. Panicle length and the number of panicles of mutant also were reduced. On the other hand, leaf length was 37% increased as compared to Koshihikari (Fig. 1E).

The mutant plants showed decreased number of florets from the base to the peak of panicles. Frequently, the florets of the panicle did not develop completely (Fig. 2A). The mutant has defects in development of floral organ such as carpel, stamen and lodicules. The lodicules were weaker than Koshihikari (Fig. 2B). The degenerated palea and lemma were unable to close, which resulted in the exposure of stamens and stigmas. In this mutant, the pistil and stamens are malformed (Fig. 2C). Especially, the anthers of the mutant were white and small (Fig. 2D). The pollen fertility was also lower than that of the Koshihikari (Fig. 2E, F). In seeds, both palea and lemma failed to close; the seeds were smaller and exposed (Grain length: 4.6 ± 0.03 cm compared with 5.1 ± 0.01 cm in the wild type; Grain width: 2.2 ± 0.05 cm compared with 3.0 ± 0.02 cm in the wild type; n=30, P<0.001) (Fig. 2G).

The representative phenotype of this mutant is leaf rolling. The anatomic analysis based on the study of transverse sections revealed that the mutant leaves had fewer veins and smaller bulliform cells comparing to those of Koshihikari. The mutant leaves were rolled while leaves in Koshihikari plants were completely flat (Fig. 3A, B). Mutant had fewer large veins (lateral veins) and small veins (intermediate veins) than those of Koshihikari, resulting in the narrow-leaf phenotypes. The analysis of cross-sections of mutant leaves showed reduced bulliform cells. The bulliform cells exist specifically in the upper side of leaves in rice, and their number and density affects determination of the leaf rolling and stretching movement (Li et al., 1979). Therefore, developmental defects of veins and bulliform cells affected the leaf rolling morphology.

For the genetic analysis of the rolled leaf phenotype, the
Fig. 3. Comparison of leaf microstructure of wild-type and rolled leaf mutant.
A. Part of a flag leaf in wild-type (left) and mutant (right)
B. Cross-section of leaves in wild-type (left) and mutant (right). Bar=1 mm.
C. Cross-section of midrib in wild-type (left) and mutant (right). Bar=100 μm.
D. Cross-section of leaf blade in wild-type (left) and mutant (right). Bar=100 μm.
E. Cross-section of large vein in wild-type (left) and mutant (right). Bar=100 μm. lv large vascular, sv small vascular, bc bulliform cells.

Table 1. Segregation ratio of the rolled leaf trait in F1 and F2 populations

<table>
<thead>
<tr>
<th>Cross combination</th>
<th>Generation</th>
<th>No. of plants</th>
<th>Total</th>
<th>X²(3:1)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Rolled leaf</td>
<td>Normal</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rolled leaf mutant (790582-1)</td>
<td>Parent</td>
<td>12</td>
<td>8</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>Rolled leaf mutant (790582-2)</td>
<td>Parent</td>
<td>17</td>
<td>10</td>
<td>27</td>
<td></td>
</tr>
<tr>
<td>790582-1/M.23</td>
<td>F1</td>
<td>7</td>
<td>10</td>
<td>17</td>
<td></td>
</tr>
<tr>
<td>790582-2/Koshihikari</td>
<td>F1</td>
<td>15</td>
<td>14</td>
<td>29</td>
<td></td>
</tr>
<tr>
<td>790582-1/M.23</td>
<td>F2 Nor</td>
<td>0</td>
<td>100</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td></td>
<td>F2 Rolled</td>
<td>81</td>
<td>78</td>
<td>159</td>
<td>49.08</td>
</tr>
<tr>
<td>790582-2/Koshihikari</td>
<td>F2 Nor</td>
<td>0</td>
<td>100</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td></td>
<td>F2 Rolled</td>
<td>114(84:30)</td>
<td>33</td>
<td>147</td>
<td>0.51</td>
</tr>
</tbody>
</table>

* Nor and Rolled: F2 plants derived from normal and rolled leaf F1 plants, respectively

Rolled leaf mutant was crossed to Milyang 23 (Tongil type) and Koshihikari (Table 1). Unexpectedly, the F1 plants from the cross between the rolled leaf mutant and Milyang 23 were segregated into 7 rolled leaf plants and 10 normal plants. The F2 plants harvested from normal F1 plants were all normal without segregation of rolled leaf plants, whereas F2 plants derived from rolled leaf F1 plants were segregated into normal and rolled leaf plants. In the seven F2 populations from the rolled leaf F1 plants, the segregation ratio didn’t fit to clear-cut ratio, which could be explained by the distorted segregation phenomenon in the inter-subspecific hybrid (Lin et al., 1992).

We also detected the segregation in the F1 plants from the cross between the rolled leaf mutant and Koshihikari (15 rolled leaf plants and 14 normal plants) and the normal F1 plant produced only normal plant in F2, whereas the rolled leaf plants produced normal plants and rolled leaf plants in F2. The F2 populations derived from the rolled leaf F1 plants derived from the cross of rolled leaf mutant / Koshihikari produced 114 rolled leaf plants and 33 normal plants, which showed the ratio of 3:1 (X²=0.51, X₀.05²= 0.48), implying that the mutant phenotype might be controlled by a single dominant gene. In addition, we found that 30 F2 plants out of 114 rolled leaf F2 plants showed abnormal growth so they died before producing seeds while 84 rolled leaf F2 plants produced F3 seeds. Segregation ratio between
the normal and abnormal growth plants of rolled leaf F₂ plants were 2:1. In addition, we have found that the mutant line was not genetically fixed despite individual selection and generation advancement for more than 3 generations as seen in the F₁ and F₂ populations. With these unusual genetic inheritance phenomenon, we hypothesize that homozygous and heterozygous in rolled leaf phenotype plants showed different growth patterns so the rolled leaf characters were maintained through heterozygous individuals and the rolled leaf phenotype is likely controlled by single dominant gene. To confirm our hypothesis, in the further study, the genetic analysis for the F₃ generation will be conducted. Also, the full sequencing analysis of the rolled leaf mutant to detect the gene(s) controlling the rolled leaf phenotype will be conducted in the further study.

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REFERENCE