Morphological Structure and Genetic Mapping of New Leaf-Color Mutant Gene in Rice (Oryza sativa)

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Abstract: Leaf-color mutations are a widely-observed class of mutations, playing an important role in the study of chlorophyll biosynthesis and plant chloroplast structure, function, genetics and development. A naturally-occurring leaf-color rice mutant, Baihuaidao 7, was analyzed. Mutant plants typically exhibited a green-white-green leaf-color progression, but this phenotype was only expressed in the presence of a stress signal induced by mechanical scarification such as transplantation. Prior to the appearance of white leaves, mutant plant growth, leaf color, chlorophyll content, and chloroplast ultrastructure appeared to be identical to those of the wild type. After the changeover to white leaf color, an examination of the mutated leaves revealed a decrease in total chlorophyll, chlorophyll a, chlorophyll b, and carotenoid content, a reduction in the number of chloroplast grana lamella and grana, and a gradual degradation of the thylakoid lamellas. At maturity, the mutant plant was etiolated and dwarfed compared with wild-type plants. Genetic analysis indicated that the leaf mutant character is controlled by a recessive nuclear gene. Genetic mapping of the mutant gene was performed using an F2 population derived from a Baihuaidao 7 × Jiangxi 1587 cross. The mutant gene was mapped to rice chromosome 11, positioned between InDel markers L59.2-7 and L64.8-11, which are separated by approximately 740.5 kb. The mutant gene is believed to be a new leaf-color mutant gene in rice, and is tentatively designated as gwgl.

Key words: Oryza sativa; leaf-color mutant; morphological structure; genetic analysis; gene mapping

Leaf-color mutations are a common type of mutations in crop plants. Mutated genes for leaf color directly or indirectly influence chlorophyll biosynthesis and biodegradation pathways, depress chlorophyll content, and affect rice photosynthesis, which lead to yield reduction and death. Leaf-color mutations are also known as chlorophyll-deficient mutations. Many chlorophyll-deficient mutants have been found in Zea mays (Lonosky et al, 2004), Pisum sativum (Highkin et al, 1969), Nicotiana tabacum (Okabe et al, 1977), Glycine max (Eric and Linda, 1994), Hordeum vulgare (Preiss and Thomber, 1995), Arabidopsis thaliana (Carol et al, 1999) and Oryza sativa (Jung et al, 2003), and they play an important role in the study of plant photosynthesis, chlorophyll biosynthesis, structure and function, genetic regulation of chloroplast development, as well as in genetic breeding (Dong et al, 1995; Larkin et al, 2003). Whole genome sequencing of Oryza sativa, a monocot model plant, has greatly facilitated functional genomic study in rice. The use of rice leaf-color mutants to discover genes that induce chlorophyll deficiency may assist genetic breeding experiments in rice after further localization, cloning and functional analysis.

Leaf-color mutant characters are normally controlled by recessive nuclear genes, with only a few cases of control by dominant or cytoplasmic genes (Qian et al, 1996; Li et al, 2002). For example, rice albino genes all-all0 proved to be recessive (Iwata et al, 1978). Other examples of leaf-color mutants under the control of recessive nuclear genes include D83 (yellow-green leaf mutant) (Li et al, 2010), pgl2 (thermo-insensitive pale green leaf mutant) (Zhu et al, 2007), st9(t) (stripe) and chll2(t) (chlorophyll-deficit) (Zhang et al, 2010), and gws (temperature-sensitive green-white-stripe leaf mutant) (Xu et al, 2010). Leaf-color mutants controlled by dominant nuclear and cytoplasmic genes are relatively uncommon. Li et al (2002) reported a dark-green rice mutant controlled by a dominant nuclear gene, and Qian et al (1996) found a white-green mutant in F1 generation of Xiushui 11 × Chunjiang 03, which was controlled by cytoplasmic genes. Although natural and induced chlorophyll-deficient mutants have been studied from different perspectives using a variety of approaches, including chlorophyll content and composition, photosynthesis capacity, chlorophyll fluorescence characteristics,
chloroplast precursor substances, chloroplast ultrastructure, thylakoid membrane proteins, mutation characteristic heredity, mutation gene mapping and mutation mechanism, experiments have often yielded conflicting results (Zhao et al., 2001; Xu et al., 2006; Wu et al., 2007; Liu et al., 2007; Huang et al., 2008).

In this study, we reported a new, naturally-occurring leaf-color mutant of rice, and focused on its agronomic traits, chlorophyll content, and chloroplast ultrastructure. Genetic analysis and mapping of the mutant gene were carried out to lay the foundation for cloning, functional studies, and breeding applications with related genes.

**MATERIALS AND METHODS**

**Materials**

Plants of the new naturally-occurring leaf-color mutant Baihuaidao 7 and its wild-type counterpart Huaidao 7 were used as experimental materials. Populations used for genetic analysis were F1, F2 and BC1F1 generations derived from Baihuaidao 7 × Jiangxi 1587 and Baihuaidao 7 × Pei’ai 64 crosses. For preliminary mapping of the mutant gene, we used an F2 population arising from Baihuaidao 7 × Jiangxi 1587.

**Main agronomic traits investigated**

Plant height, panicle number per plant, panicle length, total grain number per plant, filled grain number per plant, seed-setting rate, and 1000-grain weight were investigated in mature mutant and wild-type plants.

**Photosynthetic pigment content determination**

Leaves were collected in an identical order from wild type and white-leaf mutant seedlings. Their photosynthetic pigment content was determined following procedures outlined in Tang et al. (2004).

**Preparation and observation of chloroplast specimens under electron microscopy**

Detached leaves from white-leaf mutant and wild type seedlings were prepared and observed according to the method of Lu et al. (2009).

**Extraction of rice genomic DNA and PCR amplification**

Genomic DNA was extracted using the CTAB method (Murray and Thompson, 1980) with minor modifications. PCR amplification was performed as described by Wu and Tanksley (1993) and the resulting products were checked by agarose gel (3%–4%) electrophoresis.

**Selection of SSR markers**

A total of 475 SSR markers distributed on the 12 rice chromosomes were selected from the GRAMENE database (www.gramene.org). The primers for the markers were synthesized by the Sangon Biological Engineering Technology and Service Co. (Shanghai, China).

**Development of InDel markers**

On the basis of polymorphisms detected by SSR markers and preliminary localization of the mutant gene, we compared the clone sequences of japonica rice Nipponbare (http://rgp.dna.affrc.go.jp/) and genome sequences of Indica 9311 (http://www.btn.genomic.org.cn/rice) using NCBI BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) to detect sequence differences between these two rice varieties due to the presence of insertions and deletions (InDels). Based on these InDel sequences, primers were designed with Primer 5.0 (http://www.tucows.com/preview/205452), and were synthesized by the Sangon Biological Engineering Technology and Service Co. (Shanghai, China) (Ma et al., 2008).

**Determination of physical distance between molecular markers**

Determination of physical distance between molecular markers followed the method of Ma et al. (2008).

**RESULTS**

**Characterization of novel leaf-color mutant**

Leaves of the novel leaf-color mutant exhibited a green-white-green transformation. During the nursery period, the leaf color of Baihuaidao 7 was normal green, as shown in Fig. 1-A. Although no color variation was observed before transplanting, developed leaves turned white at 7 d after transplanting. New leaves as well as sheaths exhibited this whitening phenomenon (Fig. 1-B and -C). Fifteen days after transplanting, newly-appearing leaves returned green (Fig. 1-D). Compared with wild type plants, mutants at maturity exhibited shorter plant heights and yellower leaves (Fig. 1-E).

**Main agronomic traits of mutant plants**

Agronomic traits at maturity, including plant height and leaf color described above (Fig. 1-E), are listed in Table 1. Average plant height of the mutant was 71.0
cm, which was 20.5 cm shorter than that of the wild type. Compared with the wild type, panicle number per plant, panicle length, total grain number per plant, filled grain number per plant, and seed-setting rate of the mutant were reduced to various extents. The only exception was the 1000-grain weight of the mutant, which was 0.8 g heavier than that of the wild type. A t-test revealed no significant difference between mutant and wild type with respect to agronomic traits, other than plant height. Leaf color variation only affected biological characters, and had no obvious impact on agronomic ones.

**Photosynthetic pigment content of mutant plants**

Prior to transplanting, the chlorophyll a content of mutant seedlings differed from that of the wild type, but was not significant. The contents of total chlorophyll and chlorophyll b in the leaves of the mutant were 10.8% and 44.4% higher than in the wild type, respectively. The carotenoid content was 69.8% lower than that of the wild type. Moreover, the content ratios of chlorophyll a to chlorophyll b and carotenoid to total chlorophyll in the mutant were 31.8% and 74.15% lower, respectively, than in the wild type (Table 2). These data indicate that when the leaf color of the mutant appeared normal at the nursery stage, its chlorophyll biosynthesis had changed dramatically. After transplanting, when the leaf color of the mutant whitened, the total chlorophyll, chlorophyll a, chlorophyll b and carotenoid contents in its leaves were significantly lower than in the wild type, but the content ratios of chlorophyll a to chlorophyll b and carotenoid to chlorophyll were generally higher. Prior to and after leaf whitening, the mutant followed

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**Table 1. Comparison of mutant and wild-type agronomic traits.**

<table>
<thead>
<tr>
<th>Agronomic trait</th>
<th>Mutant</th>
<th>Wild type</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plant height (cm)</td>
<td>71.0 ± 0.5**</td>
<td>91.5 ± 0.7</td>
</tr>
<tr>
<td>Panicle number per plant</td>
<td>10.2 ± 0.7</td>
<td>11.2 ± 0.7</td>
</tr>
<tr>
<td>Panicle length (cm)</td>
<td>14.3 ± 0.5</td>
<td>14.9 ± 0.4</td>
</tr>
<tr>
<td>Total grain number per plant</td>
<td>121.9 ± 1.1</td>
<td>145.0 ± 0.3</td>
</tr>
<tr>
<td>Filled grain number per plant</td>
<td>101.7 ± 0.5</td>
<td>128.7 ± 0.3</td>
</tr>
<tr>
<td>Seed-setting rate (%)</td>
<td>83.4 ± 0.0</td>
<td>88.8 ± 0.0</td>
</tr>
<tr>
<td>1000-grain weight (g)</td>
<td>24.3 ± 0.1</td>
<td>23.5 ± 0.3</td>
</tr>
</tbody>
</table>

**, Significant at P < 0.01 by t-test.

**Table 2. Content and relative ratio of various photosynthetic pigments in mutant and wild-type leaves.**

<table>
<thead>
<tr>
<th>Growth stage</th>
<th>Material</th>
<th>Total Chl</th>
<th>Chl a</th>
<th>Chl b</th>
<th>Car</th>
<th>Chl a/Chl b</th>
<th>Car/Chl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nursery period</td>
<td>Mutant</td>
<td>2.56 ± 0.04</td>
<td>1.66 ± 0.01</td>
<td>0.91 ± 0.05</td>
<td>0.19 ± 0.02</td>
<td>1.82 ± 0.11</td>
<td>0.07 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>Wild</td>
<td>2.31 ± 0.06</td>
<td>1.68 ± 0.02</td>
<td>0.63 ± 0.09</td>
<td>0.63 ± 0.07</td>
<td>2.67 ± 0.32</td>
<td>0.27 ± 0.01</td>
</tr>
<tr>
<td>After transplanting</td>
<td>Mutant</td>
<td>0.23 ± 0.08</td>
<td>0.18 ± 0.06</td>
<td>0.05 ± 0.01</td>
<td>0.05 ± 0.01</td>
<td>3.60 ± 0.23</td>
<td>0.22 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>Wild</td>
<td>2.77 ± 0.01</td>
<td>1.61 ± 0.01</td>
<td>1.15 ± 0.01</td>
<td>0.08 ± 0.07</td>
<td>1.40 ± 0.16</td>
<td>0.03 ± 0.00</td>
</tr>
</tbody>
</table>

Chl, Chlorophyll; Car, Carotenoid.
the same trend in the changes of photosynthetic pigment content of the wild type, indicating that amounts of total chlorophyll and chlorophyll b declined greatly.

**Electron microscopic observation of chloroplasts from mutant plant**

Prior to and after leaf whitening, leaves of the mutants and wild types were extracted to examine chloroplast ultrastructure. During the nursery stage, there were no significant differences in chloroplasts from the two plant types. Mutant and wild type chloroplasts were both found to be elliptical and closely-spaced, with numerous tightly-packed grana and a grumous substrate (Fig. 2-A and -B). Therefore, chloroplast development in the mutants was normal. Following transplantation, however, the mutant leaves turned white and, in the chloroplasts, thylakoids gradually degraded and grana became more loosely-packed and significantly reduced in number (Fig. 2-C). The leaf color variation of the mutant was thus related to chloroplast developmental anomalies.

**Genetic analysis of mutant gene**

Leaf color variation was not observed in the offspring of reciprocal crosses between the mutant and Jiangxi 1587 or Pei’ai 64, confirming that the mutant trait was controlled by recessive nuclear gene, without cytoplasmic effects. In the two BC1F1 populations arising from the cross of Baihuaidao 7 with F1 generations of Baihuaidao 7 × Jiangxi 1587 and Baihuaidao 7 × Pei’ai 64, the segregation ratio of normal plants to mutants was close to 1:1 (Table 3). These results indicate that the mutant character is controlled by a recessive nuclear gene. In the F2 population of Baihuaidao 7 × Jiangxi 1587, the numbers of plants with normal and mutated leaf color were 612 and 148, respectively, and the segregation ratio of normal plants to mutants approached 3:1 ($\chi^2 = 3.38 < \chi^2_{0.05} = 3.84$), using the $\chi^2$ test. These results again indicate that the mutant character is controlled by a recessive nuclear gene. There were, actually, fewer mutant offspring than expected, as the segregation ratio of normal plants to mutants was greater than 3:1. This skewed ratio may have arisen from the genetic backgrounds of the two parents: the mutant is a late-maturing japonica rice, whereas Jiangxi 1587 is an early-season rice.

**Preliminary mapping of mutant gene**

To determine on which chromosome the target gene resides, approximately 475 SSR markers distributed on 12 chromosomes were used to detect polymorphic loci shared by the two gene pools. These gene pools were constructed by combining 20 DNA samples selected from normal green and mutant plants in an F2 population derived from a cross between Baihuaidao 7 and Jiangxi 1587. The leaf-color mutation gene was first located on chromosome 11 and was found to be linked to SSR markers RM536 and RM287 (Fig. 3-A). Based on the genetic linkage analysis, 148 recessive plants from an F2 population generated from the cross Baihuaidao 7 × Jiangxi 1587 was used to map the mutant.

<table>
<thead>
<tr>
<th>Combination</th>
<th>Generation</th>
<th>Normal plant</th>
<th>Mutant</th>
<th>Segregation ratio</th>
<th>$\chi^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baihuaidao 7 // Baihuaidao7 / Jiangxi 1587</td>
<td>BC,F1</td>
<td>21</td>
<td>19</td>
<td>1:1</td>
<td>0.05</td>
</tr>
<tr>
<td>Baihuaidao 7 // Baihuaidao7 / Pei’ai 64</td>
<td>BC,F1</td>
<td>33</td>
<td>32</td>
<td>1:1</td>
<td>0.01</td>
</tr>
<tr>
<td>Baihuaidao 7 / Jiangxi 1587</td>
<td>$F_2$</td>
<td>612</td>
<td>148</td>
<td>3:1</td>
<td>3.38</td>
</tr>
</tbody>
</table>
gene. The gene was initially located on chromosome 11 between the InDel markers L62.0-1 and L68.4-1 (Fig. 3-B). The mutant gene was then further mapped between the InDel markers L59.2-7 and L64.8-11, which were located between L62.0-1 and L68.4-1, and separated by a physical distance of 740.5 kb (Fig. 3-C).

DISCUSSION

Leaf characteristics of the mutant

The leaf-color mutant reported in this study typically undergoes a three-stage leaf color transition of green-white-green. During the first stage, leaves of mutant seedlings are green as in normal rice. During the second stage, about 7 d after transplanting, the mutant displays a white leaf color. In the third stage, about 15 d after transplanting, newly-developing leaves are once again green in color. Rice leaf-color albino mutants have been studied previously. Yu et al (2005) reported the discovery of the albino mutant alb21, and Yang et al (2005) described another albino mutant generated from a T-DNA insertion. Most of these mutants gradually die after about one month of growth. The novel mutant in this study behaves differently. Leaf color variation never arises in the case of direct sowing or from non-transplanted seedlings. About 7 d after transplanting, the first three white leaves are etiolated; later developing leaves are normally green, but the albinic leaves do not revert to green and gradually senesce. Consequently, the characteristic of leaf color variation in this mutant is completely different from that of previously reported ones.

Strangely enough, the mutant character only appears as a result of stress induced by mechanical scarification processes such as transplantation. Leaves remain green if the seedling is not transplanted. What causes the variation of leaf color: the root injury or the change in rhizosphere microenvironment? During transplanting, the seedling root is typically disturbed, but when we altered the nutrient composition of the water used at planting and performed root pruning, the leaf color of the mutant did not vary (unpublished data). Consequently, we cannot conclude that root injury directly triggered the leaf color variation. The exact cause cannot be determined and worthy of further exploration. We propose that a stress signal induced by mechanical injury, such as transplantation or change in the rhizosphere affects the nutrient transportation pathway essential to chloroplast development. Because photosynthetic products preferentially support the adult plant’s growth, and chloroplast development will be inhibited, leading to an albino leaf color. Once the stress signal from mechanical injury is removed, or the rhizosphere returns to normal, the nutrient transportation pathway necessary for chloroplast development would be recovered; once chloroplast development returns to normal, developing new leaves will also be green in color. This hypothesis, however, needs to be confirmed by major physiological experiments. Nevertheless, leaf color variation in a transplanted mutant, arising from the stress signal triggered either by mechanical injury during transplantation or from the changed root microenvironment, has not been previously reported in studies involving leaf-color mutants.

In summary, this newly-reported mutant possesses a novel leaf phenotype and a new leaf variation mechanism. Consequently, this mutant would be an ideal model system for studies on organization, function, and mechanisms of photosynthesis. In pursuit of this goal, research is continuing on the genetics, gene function, and physiological characteristics of the mutant.

Photosynthetic pigment content and microstructure of the mutant

Chlorophyll is the primary photosynthetic pigment in plants, and changes in chlorophyll content affect plant leaf color. The chloroplast is the site of plant photosynthesis, within which the absorption, transmission,
and conversion of light energy in photosynthesis take place in the thylakoid membrane. In the chloroplasts of higher plants, thylakoid membranes are not scattered randomly; they generally stack and fold to form grana. The structure of the thylakoid membrane enables it to capture light energy, optimizing the efficiency of light quantum absorption and enhancing transfer efficiency of light energy in photosynthesis. Although various changes to thylakoid membrane structure have been reported for different mutants, research in many cases has also discovered changes in chlorophyll content and the chloroplast internal structure of chlorophyll-deficit mutants. For instance, the altered phenotypes of yellow-green leaf mutant $D83$ (Li et al, 2010) and thermo-insensitive pale green leaf mutant $pgl2$ (Zhu et al, 2007) are related to chlorophyll content. In addition, in rice leaf-color mutants $ygl5$ and $pygl$ (Lu et al, 2009), white stripe leaf mutant $WG20$, and yellow leaf mutant $YZ138$ (Zhang et al, 2010), mutated phenotypes were always related to chlorophyll content and chloroplast internal structure.

When chlorophyll content was measured before and after leaf whitening, we found the total chlorophyll, chlorophyll a, chlorophyll b, and carotenoid content in the mutant was much lower after the color change than before, and also in comparison with the wild type. At the same time, chloroplast ultrastructure of the mutant was obviously different before and after leaf-color whitening. Chlorophyll development of the mutant must therefore be anomalous following leaf color whitening. These results indicate that leaf color variation is related to photosynthetic pigment synthesis and chloroplast organizational and developmental anomalies.

**Genetic analysis and molecular mapping of mutant gene**

Genetic analysis of F$_1$ and BC$_1$F$_1$ populations derived from Baihuaidao 7 back-crossed with an F$_1$ generation from Baihuaidao 7 × Jiangxi 1587 revealed that the character of the novel rice leaf-color mutant was controlled by a recessive nuclear gene. Genetic analysis of the F$_2$ population from a Baihuaidao 7 × Jiangxi 1587 cross also indicated that the mutated character was controlled by a recessive nuclear gene. Offspring with recessive phenotypes were infrequent, however, resulting in a large segregation ratio. This may be due to the great difference between the two parents’ growth period, reducing the number of recessive plants and distorting the japonica > indica hybrid ratio.

Prior to this study, several chlorophyll-deficit rice mutant genes had been mapped onto rice chromosome 11, including thermo-sensitive leaf-color mutant tsc1(t), green-revertible albino v4 and v9, and leaf spot mutants z1 and z2. Because no leaf-color mutant genes have previously been reported from the region of chromosome 11 corresponding to our new leaf-color mutant, we believe the gene reported here is a new gene, which we tentatively named $gwgl$. The mutant gene was mapped onto rice chromosome 11 between InDel markers L59.2-7 and L64.8-11, which are separated by a distance of approximately 740.5 kb. The markers L64.2-2 and L64.8-10, located between the markers L59.2-7 and L64.8-11, were developed, but no recombinant plants were found. A larger population is thus needed for fine-mapping of the mutant gene. The preliminary mapping and newly-developed markers in this study will serve as a future foundation for fine-mapping, cloning, and functional analysis of the mutant gene.

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