Genetic Analysis and Molecular Mapping of Novel White Striped Leaf Mutant Gene in Rice

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Abstract: A new white striped leaf mutant wsl1 was discovered from Nipponbare mutated by ethyl methanesulfonate. The mutant showed white striped leaves at the seedling stage and the leaves gradually turned green after the tillering stage. The chlorophyll content of wsl1 was significantly lower than that of wild-type during the fourth leaf stage, tillering stage and booting stage. The numbers of chloroplast, grana and grana lamella were reduced and the thylakoids were degenerated in wsl1 compared with wild type. Genetic analysis showed that the wsl1 was controlled by a single recessive gene. Molecular mapping of the wsl1 was performed using an F2 population derived from wsl1/Nanjing 11. The wsl1 was finally mapped on the telomere region of chromosome 9 and positioned between simple sequence repeat markers RM23742 and RM23759 which are separated by approximately 486.5 kb. The results may facilitate map-based cloning of wsl1 and understanding of the molecular mechanism of the regulation of leaf-color by WSL1 in rice.

Key words: rice; white striped leaf mutant; genetic analysis; gene mapping

Chlorophyll plays unique and indispensable roles in metabolism and energy transfer in the reaction centers of photosynthesis and is of great importance to all algae, bacteria and plants in relation to photosynthesis (Liu et al, 2007). Nearly 10^9 t of chlorophyll are synthesized and degraded on our globe each year (Eckhardt et al, 2004). Rice provides the staple food for nearly half of the world’s population, and is the model monocot plant for gene function analysis (Delseny et al, 2001). Rice leaf-color mutation is common in crop plants. The mutated genes for leaf-color directly or indirectly influence chlorophyll biosynthesis and biodegradation pathways, depress chlorophyll content, and further affect rice photosynthesis, which lead to yield reduction and even plant death. Therefore, the research on rice leaf-color mutations is of great importance in clarifying the photosynthesis mechanism of chloroplast and in rice breeding with high efficiency of photosynthesis.

Leaf-color mutants involving chlorophyll deficiency are excellent materials in the research of chlorophyll biosynthesis and chloroplast development. Numbers of such mutants have been identified in higher plants such as Arabidopsis (Hoeberechts et al, 2008), sunflower (Yue et al, 2009), sweetclover (Bevins et al, 1993), barley (Liu et al, 2008) and maize (Pasini et al, 2005). The chlorophyll metabolic pathway has been defined by identifying the major genes of the process in Arabidopsis (Beale, 2005). At least 70 leaf-color mutants have been identified in rice (Huang et al, 2005; Chen et al, 2007), most of which are normally controlled by recessive nuclear genes, with only a few cases controlled by dominant or cytoplasmic genes (Qian et al, 1996; Li et al, 2012). For example, the rice albino genes nal (Iwata et al, 1978), d83 (yellow-green leaf mutant) (Li et al, 2010), pgl2 (thermosensitive pale green leaf mutant) (Zhu et al, 2007), st9(t) (stripe), chill2(t) (chlorophyll-deficit) (Zhang et al, 2010) and gws (temperature-sensitive green-white-stripe leaf mutant) (Xu et al, 2010) are all controlled by recessive nuclear genes. Although numbers of rice leaf-color mutant genes have been mapped and some even cloned, the molecular mechanism and regulation pathway of chlorophyll metabolism in the mutants still need further research.

In the present study, a new rice leaf-color mutant was discovered from Nipponbare. The agronomic traits,
chlorophyll content and chloroplast ultrastructure have been investigated. The genetic analysis indicated that the white striped phenotype of \textit{wsl1} mutant was controlled by a single recessive gene that was mapped on the telomere region of chromosome 9. These results could be used to map-based cloning of \textit{wsl1} and to understand the molecular mechanism of the regulation of leaf-color by \textit{WSL1} in rice.

**MATERIALS AND METHODS**

**Plant materials**

The white striped leaf mutant \textit{wsl1}, mutated from Nipponbare by ethyl methanesulfonate (EMS), was inherited stably after three generations of self-crossing. Two \(F_2\) populations generated from the crosses of \textit{wsl1}/Pei’ai 64 and \textit{wsl1}/Nanjing 11 were used for genetical analysis of the \textit{wsl1} gene. Then an \(F_2\) population of 35 000 individuals derived from the cross of \textit{wsl1}/Nanjing 11 was used to fine map the \textit{wsl1} gene. All the plant materials were grown in the experimental fields of China National Rice Research Institute, Hangzhou, Zhejiang Province.

**Investigation of agronomic traits**

Plant height, panicle length, total grain number per plant, filled grain number per plant, seed-setting rate and 1000-grain weight were investigated in \textit{wsl1} and its wild type plants after maturity.

**Detection of chlorophyll content and chloroplast ultrastructure**

During the seedling, tillering, booting and heading stages, the chlorophyll content of \textit{wsl1} and its wild type Nipponbare was measured. The extraction, measurement and calculation were conducted according to the methods of Tang et al (2004).

Chloroplast ultrastructure was examined using leaves from \textit{wsl1} and its wild type during the tillering stages. The leaves were fixed in a solution of 2% glutaraldehyde and further fixed in 1% OsO\textsubscript{4}. After staining with uranyl acetate, tissues were further dehydrated in an ethanol series, and finally embedded in Spurr’s medium prior to ultrathinsectioning. Samples were stained again and examined with a Hitachi H-7650 transmission electron microscope (Hitachi 7650) (Lu et al, 2009).

**Genomic DNA extraction and genotypic analysis**

Genomic DNA was extracted from fresh-frozen leaves of each white striped individual in \(F_2\) population from \textit{wsl1}/Nanjing 11 by using the SDS method (Zhu et al, 2009). The extracted DNA was dissolved in ddH\textsubscript{2}O. A total of 1 051 SSR markers (http://www.gramene.org/) covering 12 rice chromosomes were used to detect the polymorphism between the two parents (\textit{wsl1} and Nanjing 11). Polymerase chain reaction (PCR) was performed in a 10-\(\mu\)L reaction volume containing 25 ng of template DNA, 1.0 \(\mu\)L of 10 \(\times\) PCR buffer, 0.1 mmol/L dNTP, 0.1 \(\mu\)mol/L primer pairs, and 0.1 U \textit{Taq} DNA polymerase. The amplification protocol included an initial denaturation at 95 °C for 3 min, followed by 35 cycles of 30 s denaturation at 95 °C, 30 s annealing at 55 °C, and extension of 40 s at 72 °C, and a final extension step of 8 min at 72 °C. The PCR products were electrophoretically separated through a 6% native polyacrylamide gel, and the amplified DNA fragments were silver-stained for visualization (Xu et al, 2010).

**RESULTS**

**Phenotype identification of \textit{wsl1}**

The \textit{wsl1} mutant exhibited distinct white striped leaves during the seedling and tillering stages (Fig. 1). However, the white striped leaf phenotype gradually restored green after tillering with the temperature rising. At maturity, the plant height of the \textit{wsl1} mutant was significantly shorter than that of the wild type. While the other agronomic characters showed no significant difference between \textit{wsl1} and its wild type (Table 1).

**Measurement and analysis of chlorophyll content**

With the development of \textit{wsl1} and its wild type, the

![Fig. 1. Phenotypes of \textit{wsl1} and its wild type plant during seedling and tillering stages.](image-url)

A, Seedling of \textit{wsl1} (right) and its wild type (left); B, Phenotype of \textit{wsl1} (right) and wild type (left) at the tillering stage; C, Leaf of \textit{wsl1} (right) and wild type (left) at the tillering stage.
contents of chlorophyll a (Chla), chlorophyll b (Chlb) and the total chlorophyll were all increased, but were all lower in 
\textit{wsl1} than in the wild type. The Chla content, Chlb content and the total chlorophyll content of 
\textit{wsl1} at the heading stage (1.36 mg/g, 1.22 mg/g and 2.58 mg/g) were just slightly lower than those of 
its wild type (1.47 mg/g, 1.34 mg/g and 2.81 mg/g). (Fig. 2). With the development of 
\textit{wsl1} and its wild 
type, the ratio of Chla/Chlb was reduced, while the 
ratio of Chla/Chlb in 
\textit{wsl1} was higher than that in the 
wild type at the seedling, tillering and booting stages. However, at the heading stage, the ratio of Chla/Chlb 
of \textit{wsl1} (1.12) was nearly the same as that of its wild 
type (1.10) (Fig. 2).

**Chloroplast ultrastructure examination**

To investigate how the \textit{wsl1} mutation affects chloroplast development, we compared the ultrastructures of chloroplasts in the \textit{wsl1} and wild-type plants using the transmission electron microscopy (Hitachi 7650). During the tillering stage, the number of chloroplasts in the white part of \textit{wsl1} leaves was much less than that in its wild type (Fig. 3-A and -D), the thylakoids were vague and degraded, and the grana lamellar became more loosely-packed, discontinued and significantly reduced in number (Fig. 3-A to -F). In addition, the number of osmium corpuscles in the chloroplasts of \textit{wsl1} was much more than that of its wild type, while the number of starch granules was significantly reduced (Fig. 3-D to -I).

Within the green part of \textit{wsl1}, the leaf color showed light green, and the number of chloroplasts in the mesophyll cell was increased (Fig. 3-A, -D and -G), and there also were some chloroplasts with well-developed grana lamellar structure similar to those of the wild-type in the mesophyll cells of \textit{wsl1} (Fig. 3-G to -I).

**Genetic analysis of \textit{wsl1}**

For genetic analysis of \textit{wsl1}, two F\textsubscript{2} populations were constructed from the crosses of \textit{wsl1}/Pei’ai 64 and \textit{wsl1}/Nanjing 11. All F\textsubscript{1} plants from the two crosses displayed wild-type phenotype, and their F\textsubscript{2} progenies all showed a segregation ratio of 3:1 for green and white striped plants (Table 2). Therefore, the mutant phenotype of \textit{wsl1} was controlled by a single recessive nuclear gene.

**Molecular mapping of \textit{wsl1}**

Among the 1 051 SSR markers covering 12 rice chromosomes, 363 SSR markers showed polymorphisms between the two parents \textit{wsl1} and Nanjing 11. The

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### Table 1. Comparison of main agronomic characters between \textit{wsl1} and its wild-type.

<table>
<thead>
<tr>
<th>Agronomic trait</th>
<th>Mutant</th>
<th>Wild type</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plant height (cm)</td>
<td>89.2 ± 2.0</td>
<td>97.9 ± 3.6*</td>
</tr>
<tr>
<td>Panicle length (cm)</td>
<td>21.5 ± 1.0</td>
<td>22.5 ± 0.8</td>
</tr>
<tr>
<td>Flag leaf length (cm)</td>
<td>31.1 ± 3.5</td>
<td>32.2 ± 2.7</td>
</tr>
<tr>
<td>Flag leaf width (cm)</td>
<td>1.2 ± 0.1</td>
<td>1.3 ± 0.1</td>
</tr>
<tr>
<td>Grain length (cm)</td>
<td>0.7 ± 0.1</td>
<td>0.8 ± 0.1</td>
</tr>
<tr>
<td>Grain width (cm)</td>
<td>0.3 ± 0.1</td>
<td>0.4 ± 0.1</td>
</tr>
<tr>
<td>1000-grain weight (g)</td>
<td>24.6 ± 0.2</td>
<td>25.5 ± 0.3</td>
</tr>
<tr>
<td>Total grain number per panicle</td>
<td>129.0 ± 18.0</td>
<td>133.0 ± 8.8</td>
</tr>
<tr>
<td>Filled grain number per panicle</td>
<td>113.0 ± 18.1</td>
<td>119.0 ± 9.5</td>
</tr>
<tr>
<td>Seed-setting rate (%)</td>
<td>85.7 ± 4.0</td>
<td>89.6 ± 3.5</td>
</tr>
</tbody>
</table>

* indicate significant difference between the trait of \textit{wsl1} and wild type (t-test, P < 0.05).

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**Fig. 2.** Chlorophyll content of \textit{wsl1} mutant during different development stages compared with that of wild type.

Chl, Chlorophyll; WT, Wild type.
linkage analysis indicated that the genotypes of five markers on the telomere region of chromosome 9 associated with the white striped phenotype of the recessive individuals from the F2 population of \textit{wsl1}/Nanjing 11. Then the five polymorphic markers RM23654, RM23662, RM23769, RM23791 and RM23828 were further employed to construct the primary linkage map of the target region. Finally, the target gene was located between the SSR markers RM23662 and RM23769 (Fig. 4-A).

In order to further fine mapping \textit{wsl1}, a larger \textit{wsl1}/Nanjing 11 F2 population containing nearly 35 000 individuals were constructed, and 20 SSR markers (http://www.gramene.org/) were developed between RM23662 and RM23769. The polymorphism analysis of the SSR markers indicated that 7 out of the 20 developed SSR markers showed polymorphisms between the two parents, then the seven polymorphic markers were further used to analyze the white striped leaf recessive individuals (Table 3). Finally, the \textit{wsl1} was limited between RM23742 and RM23759 where the physical distance was about 486.5 kb (Fig. 4-B) and possessed five BAC clones B1106C08, B1103C04, OSJNBa0017O03, OJ1178_D01 and OSJNBa0038I09 (Fig. 4-C).

**DISCUSSION**

Recently, numbers of chlorophyll and chloroplast associated mutations that affect leaf coloration have been identified and are referred to as \textit{virescent} (v), \textit{stripe} (st), \textit{albino}, \textit{chlorina}, \textit{zebra}, and \textit{yellow variegated}.
depending on their diverse phenotypes (Yoo et al., 2009). In our study, the \textit{wsl1} mutant showed white striped leaves at the seedling and tillering stages, then the white striped leaf gradually restored green until the maturity stage along with the temperature rising. Therefore, \textit{wsl1} in the study may be a temperature-conditional chlorophyll-deficient rice mutant, and further identification of temperature-conditional character should be carried out.

As is known, numbers of leaf-color mutant genes were mapped and most of them have been cloned. \textit{OsCHLH9} (Goh et al., 2004), \textit{Chlorina-1}, \textit{Chlorina-9} (Zhang et al., 2006), \textit{OsDVR} (Wang et al., 2010), \textit{virescent 1} and \textit{virescent 2} (Hiroki et al., 2004) were all located on chromosome 3; \textit{YGL1} (Wu et al., 2007), \textit{OsHAP3B} and \textit{OsHAP3C} (Kazumaru et al., 2003) were delimited on chromosome 5; \textit{NYC3} (Ryouhei et al., 2009), \textit{virescent 3} (Yoo et al., 2009) and \textit{SPP} (Yue et al., 2010) were mapped on chromosome 6; \textit{OsPPR1}, \textit{sgr} (Kodiveri et al., 2005) and \textit{cisc(t)} (Lan et al., 2007) were delimited on chromosome 9. In this study, the white striped leaf mutant gene \textit{wsl1} was located in the telomere region of chromosome 9, which was different from those of \textit{OsPPR1}, \textit{sgr} and \textit{cisc(t)}, so \textit{wsl1} might be a new rice leaf-color gene. However, the chromosome exchange frequency is quite low in the telomere region during meiosis, which brings about a big difficulty for the fine mapping of \textit{wsl1}. In this research, nearly 35 000 individuals of \textit{wsl1}/Nanjing 11 \textit{F}_{2} population were used to fine-map \textit{wsl1}, and the \textit{wsl1}

![Fig. 4. Molecular mapping of wsl1 on rice chromosome 9.](image)

A, Linkage analysis of \textit{wsl1} on chromosome 9; B, Preliminary mapping of \textit{wsl1}; C, The BAC clones among the mapping interval. \textit{n}, Number of the white striped leaf recessive individuals; Chr, Chromosome.

<table>
<thead>
<tr>
<th>Marker</th>
<th>Forward primer (5’–3’)</th>
<th>Reverse primer (5’–3’)</th>
</tr>
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<tbody>
<tr>
<td>RM23662</td>
<td>GAGAGGACGAGTAGGCACACTTTTG</td>
<td>CGAGGAACTTGATTCGACATG</td>
</tr>
<tr>
<td>RM23686</td>
<td>CCTAATGCGTGCTCCTCAACCC</td>
<td>GAGCTGTCGCCTCACTAAATAGTC</td>
</tr>
<tr>
<td>RM23714</td>
<td>GGTGATGATGATGATGATGCTG</td>
<td>TGACAGTAGTAGTCAGCATGTCGTGTC</td>
</tr>
<tr>
<td>RM23729</td>
<td>TCTCTGTCTCTAGTCTGCTTCC</td>
<td>TTGTGTTGTTCTGAGTGTGTC</td>
</tr>
<tr>
<td>RM23736</td>
<td>GCCGATACCTGCATCTACCTCC</td>
<td>CCGAAAGCAACTTGATGAGACCC</td>
</tr>
<tr>
<td>RM23742</td>
<td>GTTGCTCAGGAGAAGAGAAGAAGAGAAGAGAGAGAGAG</td>
<td>GCCAAATATCAGCTCCTCCCTC</td>
</tr>
<tr>
<td>RM23759</td>
<td>TCTTGGAGAAGAACAGAACATATTG</td>
<td>TTCCCTCCTCCGACCCTTCC</td>
</tr>
<tr>
<td>RM23766</td>
<td>AATCTATGGCTGAGCCTATTACC</td>
<td>GAAACATTAAAGCCCTTCCAGTGCG</td>
</tr>
<tr>
<td>RM23769</td>
<td>AAGAGGATATGGAGAAGAGGATG</td>
<td>ACAACCCACCCACGTCCTAGG</td>
</tr>
</tbody>
</table>

The markers RM23662 and RM23769 were the polymorphic markers which were used in \textit{wsl1} primary mapping. The other seven polymorphic markers were used in the further fine mapping of \textit{wsl1}.
was finally delimited in a 486.5 kb interval. The further expanding mapping populations and fine mapping of wsl1 are conducting, which will lay the foundation for the last cloning of wsl1.

Rice leaf-color mutant phenotype can be used as an early selected marker for efficient identification and elimination of false hybrids in commercial hybrid rice production (Su et al., 2012). Generally, receptor parent transferred with leaf-color marker genes would show great changes in other important agronomic characters that could reduce the application value of the receptor parent. In the present study, except for plant height, the other agronomic characters between wsl1 and its wild type have no significant differences. Therefore, wsl1 may have little impact on the other agronomic traits of the receptor parent, so that it can be better introduced into receptor parent as an early marker for much more efficient in identification and elimination of false hybrids in commercial hybrid rice production.

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