Identification and Genetic Mapping of a Lesion Mimic Mutant in Rice

MA Jian-yang1, 2, CHEN Sun-lu2, 3, ZHANG Jian-hui1, 2, DONG Yan-jun1, TENG Sheng2
(1Laboratory of Plant Genetics and Functional Gene, College of Life and Environment Science, Shanghai Normal University, Shanghai 200234, China; 2Institute of Plant Physiology & Ecology, Shanghai Institute for Biological Sciences, Chinese Academy of Sciences, Shanghai 200032, China; 3National Key Laboratory of Crop Genetics and Germplasm Enhancement, Nanjing Agricultural University, Nanjing 210095, China)

Abstract: A lesion mimic stripe mutant, designated as lms1 (lesion mimic stripe 1), was obtained from the M2 progeny of a 60Co γ-radiation treated japonica rice variety Jiahua 1. The lms1 mutant displayed propagation type lesions across the whole growth and developmental stages. Physiology and histochemistry analysis showed that the mutant exhibited a phenotype of white stripe when grown under high temperature (30 °C), and the lesion mimic caused by programmed cell death under low temperature (20 °C). The genetic analysis indicated that this lesion-mimic phenotype is controlled by a single locus recessive nuclear gene. Furthermore, by using simple sequence repeat markers and an F2 segregating population derived from two crosses of lms1 × 93-11 and lms1 × Pei’ai 64S, the lms1 gene was mapped between markers Indel1 and MM0112-4 with a physical distance of 400 kb on chromosome 6 in rice.

Key words: rice; lesion mimic mutant; gene mapping

Some plants can exhibit spontaneous spots on leaves in the absence of environmental stress, agrochemical damage, physical damage and pathogen attack. This kind of spots is similar to lesions caused by the infection of pathogens. We term this kind of mutants as lesion mimic mutants (Wang, 2005). Some lesion mimic traits are controlled by recessive genes, and some by dominant genes (Wang Z H et al., 2004). They exist in various plant species, including Arabidopsis thaliana, barley, maize, groundnuts, tobacco, sorghum, soybean and rice (Malamy et al., 1990; Dietrich et al., 1994; Buschges et al., 1997; Gray et al., 1997; Takahashi et al., 1999; Badigannavar et al., 2002). Lesion mimic mutants can be divided into two types according to the phenotype: propagation type and initiation type (Neuffer and Calver, 1975; Dangl et al., 1996; Shirasu and Schulze-Lefert, 2000). The distribution and the size of the propagation-type lesion mimics are comparatively constant, whereas the initiation-type ones are diffused to other parts of leaves and also to leaf sheaths and culms after formed. The lesion mimics in plants were mainly resulted from the programmed cell death led by hypersensitive response and the abnormal burst of reactive oxygen species in the apoptosis pathway (Ryerson and Heath, 1996; Dietrich et al., 1997; Lamb and Dixon, 1997). In addition, plant hormones and overexpression of exogenous genes took part in the lesion-mimic formation (Beffa et al., 1995; Mittler et al., 1995; Asai et al., 2000), and the environmental factors such as light, temperature and humidity also had influence on the formation of lesion mimics (Yamanouchi et al., 2002). To date, many lesion mimic mutant genes have been identified and cloned, such as Lsd1, acd2, acd11, Mod1, Cpn1 in A. thaliana; les22, lls1, RP1 in maize; mlo in barley; and spl7, spl11, OSlsd1, spl28 in rice (Collins et al., 1999; Jiang et al., 2003; Wang J J et al., 2004; Zeng et al., 2004; Wang et al., 2005; Qiao et al., 2010). Cloning of these genes will help to demonstrate the molecular mechanism of the occurrence and formation of the lesion mimics in plants, and to understand the genes involved in plant cell development and apoptosis and signaling pathways for stress tolerance of disease resistance in plants (Wu et al., 2008; Matin et al., 2010).

A lesion mimic stripe mutant (lms1) was selected from the progeny of a γ-radiation treated japonica rice variety Jiahua 1. Morphological characterization, genetic analysis and molecular mapping were conducted to the mutant, which will benefit for future cloning, functional analysis and application of this gene.

MATERIALS AND METHODS

Rice materials
The mutant lms1 was isolated from the M2 progeny of Jiahua 1, which was treated with 60Co γ-radiation.
All agronomic traits of lms1 were stable and uniform after self-pollination for several generations in Shanghai and Hainan, China.

Methods

Phenotypic observation

The characteristics of the mutant lms1, including the occurrence period, shape, colour and distribution of the spots on leaves were investigated across the whole growth period in the greenhouse. The agronomic traits such as plant height and seed-setting rate of the mutant lms1 in fields were also recorded in Hainan Province, China. To investigate the dependence of the phenotype of the lms1 mutant on growth temperature, germinated seeds of lms1 and its wild type were transferred into growth chambers (GXZ Zhineng-Type, Ningbo Jiangnan Instrument Ltd, China) with the same light intensity of 180 μmol/(m²·s) (12 h light/12 h dark) but two different temperatures (20 and 30 °C).

Ultrastructural analysis of chloroplasts of lms1 leaves

The white parts of lms1 leaves and the same parts of Jiahua 1 were sampled from 12-day-old seedlings grown under 30 °C, and fixed in the solution of 2.5% glutaraldehyde and 1% osmic acid (prepared with 0.2 mol/L phosphoric acid buffer solution, pH 7.2) at 4 °C. After 5 h, they were dehydrated by 50%, 70%, 80%, 95% and 100% ethylalcohol and acetone, respectively, then embedded into epoxy resins. After spliced, the samples were dyed with uranyl acetate, observed and photographed by a Hitachi-7650 transmission electron microscope (Hitachi).

Measurement of leaf pigment content

Chlorophyll was extracted with 80% acetone from 0.2 g fresh leaves of 20-day seedlings of the lms1 mutant and the wild type grown in the greenhouse, respectively. The extract was measured at 430, 467 and 663 nm by a spectral-photometer (BCEKMAN Coulter-DU720). Contents of total chlorophyll, chlorophyll a, chlorophyll b and total carotenoids were determined according to the method of Gao (2006). The experiments were repeated three times.

Trypan blue staining

The mutant and wild type leaves were stained with lactic acid-phenol-tryparan blue solution (2.5 mg/mL tryparan blue, 25% lactic acid, 23% water-saturated phenol and 25% glycerol) at boiling water bath for 10 min and kept at room temperature for 12 h before replacing the lactic acid-phenol-tryparan blue solution with a chloral hydrate solution (25 mg in 10 mL of H₂O) for 3–4 d for destaining (Yin et al, 2000). Then leaf tissues were observed under an SZ2-1LST stereoscope (Olympus) and photographed with a PC1200 digital camera (Canon). The experiments were repeated three times.

DAB (di amino benzidine) staining

DAB staining to directly localize H₂O₂ in rice leaf tissues was performed as described by Thordal-christansen (1997). The leaves of mutant and wild type rice were incubated in 1 mg/mL DAB (pH 3.8) and lighted at 25 °C for 8 h, then put into 96% ethanol and boiled 10 min to destain. Finally, the leaves were submerged in fresh ethanol at 25 °C for 4 h and observed under an SZ2-1LST stereo scope (Olympus) and photographed with a PC1200 digital camera (Canon). The experiment was repeated three times.

Genetic analysis and construction of mapping population

For genetic analysis, three F₂ populations were developed from the crosses between lms1 with 93-11, Pei’ai 64S and Guangzhan 63S, respectively. The population from the cross between lms1 and 93-11 was used for preliminary mapping the lms1 locus, and the population from the cross between lms1 and Pei’ai 64S was used for fine mapping of the lms1 gene. F₂ seeds of the crosses were germinated at 37 °C for 3 d, sown in plastic containers with rice paddy soil, and grown in the greenhouse for 14 d. The seedlings with mutant phenotype were transferred into the growth chambers for further observation.

Genomic DNA extraction

Genomic DNA was extracted from young leaves of each parent and F₂ individuals by the modified CTAB method (Muray and Thomason, 1980).

Mapping of lms1 gene

The polymorphism between the lms1 and 93-11 was tested using 231 pairs of SSR markers, which were released by http://www.gramene.org/. The polymorphic SSR markers were applied for genetic linkage analysis using a mapping population of 81 mutant individuals from the F₂ population. A total of 513 mutant individuals of the F₂ population from lms1 × Pei’ai 64S and new SSR markers developed by the Watson Institute of Genome Science, Zhejiang University, China (http://www.dnaresearch.oxfordjournals.org) were used for fine mapping of lms1. The volume of PCR system was 10 μL, including 100 mmol/L Tris-HCl (pH 9.0), 100 mmol/L KCl, 20 mmol/L MgSO₄, 80 mmol/L (NH₄)₂SO₄, 2.5 mmol/L dNTPs, 10 μmol/L primers, 5 U/μL Taq polymerase, 20 ng of template DNA. PCR amplification proceeded by the Bio-Rad PCR cycler and the primers
were synthesized by the Shanghai SBS Genetech Co. Ltd, China. The PCR program was as follows: 4 min initialization at 94 °C; 50 s denaturation at 94 °C, 45 s annealing at 55 °C, and 30 s extension at 72 °C for 35 cycles; 10 min final extension at 72 °C. After electrophoresis on agarose gels (2.5%–3.5%) and drying with ethidium bromide, the products were imaged on a UVP Bioimaging system.

**RESULTS**

**Phenotypes of lms1**

The rice lms1 mutant grown in the greenhouse exhibited brown-yellow stripes before the first true leaf unfolding at the seedling stage. With the leaf expansion, the stripes enlarged and grew into yellow-white streaks, where the necrotic lesion located (Fig. 1-A and 1-B). A leaf generally displayed two or three groups of lesion mimics, which were mostly located on the upper and middle parts of leaves. The lesion mimics were also observed on culms during the whole growth period as well as leaves. The mature mutants planted in the fields were dwarfed and premature senescence with lower seed-setting rate (Fig. 1-C) as compared with the wild type under the same conditions, which was also observed in the greenhouse.

To investigate the effect of temperature on the phenotype, the phenotypes of lms1 plants grown under different temperatures (20 and 30 °C) were surveyed. The wild type of Jiahua 1 had normal green leaves at two temperatures, whereas white stripes emerged on the leaves of the lms1 mutant at 30 °C, and white stripes and brown necrotic lesions appeared at 20 °C (Fig. 2), which suggests that low temperature accelerates the formation of lesion mimics in the lms1 mutant.

**Photosynthetic pigment content in lms1**

By measuring the photosynthetic pigment content in the leaves of the wild type and mutant grown in the greenhouse, we found that the total chlorophyll, chlorophyll a, chlorophyll b and carotenoid contents were decreased by 32.2%, 28.9%, 40.9% and 46.0%, respectively.
respectively (Fig. 3), suggesting that the biosynthesis of chlorophyll was interfered in the *lms1* mutant.

**Ultrastructure of leaf chloroplasts of *lms1* seedlings**

To further understand the influence on chloroplast development exerted by the mutation, a transmission electron microscope (TEM) was used to observe the chloroplasts of the wild type and *lms1*. The chloroplasts of the wild type had a characteristic structure in the ellipse shape, with normal granum composed of neatly stacked thylakoids, and the grana were intimately connected by stroma thylakoids (Fig. 4-A to 4-C). However, the chloroplasts from the white-stripe part of *lms1* leaves were aberrant. The thylakoid discs were loose and assembled into abnormal grana, which slackly connected by stroma thylakoids (Fig. 4-D to 4-F). These results indicate that the mutation of *lms1* affects the development of chloroplasts.

**Programmed cell death (PCD) and H$_2$O$_2$ accumulation in leaves of *lms1***

Trypan blue staining of leaves displayed that the leaves of *lms1* presented dark blue spots under a light blue background, indicating that a mass of PCD occurred in the leaves of *lms1* and then led to visible lesion mimics (Fig. 5-A). The emergence of dark blue spots around the lesions suggested the expansion of lesion mimics. Contrary to those of *lms1*, PCD was not observed in the leaves of wild type, since the leaves were hardly stained (Fig. 5-A). These results imply that the formation and development of *lms1* lesion mimics might be actually the process of PCD in leaves.

![Fig. 3. Comparison of the leaf chlorophyll and carotenoid contents in leaves between the *lms1* mutant and wild type.](image)

![Fig. 4. Transmission electron microscopy pictures of the chloroplasts of wild type and white-stripe part of *lms1* mutant.](image)

A, B and C, Wild type; D, E and F, *lms1* mutant.
When \( \text{H}_2\text{O}_2 \) accumulated in plant cells, the exogenous diamino benzidine (DAB) reacted with \( \text{H}_2\text{O}_2 \) via peroxidase and rapidly engendered rufous polymer, thus becoming a method for detecting the accumulation of \( \text{H}_2\text{O}_2 \). After DAB staining, the leaves from \( \text{lms1} \) appeared a great deal of rufous spots compared with those of wild type (Fig. 5-B), suggesting that \( \text{lms1} \) bred the burst of reactive oxygen species (ROS) which led to hypersensitive response.

**Genetic analysis of \( \text{lms1} \)**

The \( \text{lms1} \) mutant was crossed with three wild-type rice, Pei’ai 64S (an indica variety with the maternal origin of japonica), Guangzhan 63S (an indica sterile line) and 93-11 (a typical indica variety). All F1 plants had normal phenotypes, which indicated that the mutation was controlled by recessive nuclear genes. In the further survey of F2 generations of \( \text{lms1} \times \text{Pei’ai 64S} \) and \( \text{lms1} \times \text{Guangzhan 63S} \), the progenies of both crosses produced normal and lesion-mimic plants in a ratio of 3:1 (\( \chi^2 < \chi_{0.05}^2 = 3.84 \), Table 1). Furthermore, the 2 174 F3 progenies from the F2 generations that were heterozygous at \( \text{lms1} \) locus displayed the phenotypic segregation ratio of 3:1, namely 1 622 normal plants and 552 mutational plants (\( \chi^2 < \chi_{0.05}^2 = 3.84 \)). Hence, our statistical results suggest that the lesion-mimic phenotype is controlled by a single locus recessive nuclear gene.

**Gene mapping of \( \text{lms1} \)**

Out of 231 simple sequence repeat (SSR) markers from the Gramene Genome Browser (http://www.gramene.org), which cover 12 rice chromosomes, 95 were polymorphic between \( \text{lms1} \) and 93-11. Eighty-one mutant plants showing lesion-mimic phenotype were obtained in the F2 generation of \( \text{lms1} \times \text{93-11} \), and were then used for linkage analysis. Consequently, the \( \text{lms1} \) locus was delimited between RM469 and MM0135 on chromosome 6. Subsequently, by using of a newly-developed SSR marker MM0112-4 (5’T-CCTGGAGC AACTGTGGTA-3’, 5’T-GCCAGATAAAAGTATGGA ATG-3’) and an Indel marker Indel1 (5’T-GTAACCCC TATCCCTATGAGTATC-3’, 5’T-TTCCTTCTCTGGTAC AGCTCCTTC-3’), and 513 mutant plants of the F2 generation of \( \text{lms1} \times \text{Pei’ai 64S} \), the \( \text{lms1} \) locus was further identified within 400 kb between Indel1 and MM0112-4 with genetic distances of 2.5 and 0.7 cM, respectively (Fig. 6).

**DISCUSSION**

Natural and artificial lesion mimic mutants in plants have been one of the research hotspots in plant physiology, developmental biology, molecular biology, agronomy, and so on. The identification and functional characterization of lesion mimic related genes from the mutants facilitate the elucidation of mechanisms underlying PCD in plants (Huang et al, 2010). Recently, a number of lesion-mimic related genes were located and cloned, such as \( \text{spl7} \), the first cloned lesion-mimic gene using map-based method, which encodes a transcription factor involved in the heat shock response in rice (Yamanouchi et al, 2002). \( \text{spl1} \),

<table>
<thead>
<tr>
<th>Cross</th>
<th>Number of total plants</th>
<th>Number of mutant plants</th>
<th>Number of wild-type plants</th>
<th>( \chi^2 ) (3:1)</th>
<th>( P )</th>
</tr>
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<tbody>
<tr>
<td>( \text{lms1} \times \text{Pei’ai 64S} )</td>
<td>405</td>
<td>111</td>
<td>294</td>
<td>1.25</td>
<td>0.25−0.50</td>
</tr>
<tr>
<td>( \text{lms1} \times \text{Guangzhan 63S} )</td>
<td>1260</td>
<td>291</td>
<td>969</td>
<td>2.44</td>
<td>0.10−0.25</td>
</tr>
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cloned by Zeng et al (2004), plays a role of negative feedback regulation in cell apoptosis and defense response of rice. The formation of plant lesion mimic correlated with the regulatory disturbance of metabolic pathway led by related genes mutation, and associated with various environmental factors, including light, temperature, humidity and nutrition. The expression of necrotic leaf stripes was observed to be temperature sensitive in the lesion mimic mutant Lesl of maize (Zea mays) (Hoisington et al, 1982). Low temperature stimulates the production of necrotic phenotype in a maize lesion-mimic mutant rpl (Hu et al, 1996), and light is also noticed to regulate its disease lesion mimicry (Jodon, 1957; Nagao et al, 1964).

The lesion mimic phenotype of lms1 is radically differed from those of other three lesion-mimic mutants of rice [bl2 (Jodon, 1957; Nagao et al, 1964), bl3 (Nagao et al, 1964, 1966), spl4 (Mizobuchi R et al, 2002; Mizobuchi K et al, 2003)], whose loci were also identified on rice chromosome 6. Furthermore, the lms1 locus is located on the distal end of the short arm, and differs from the other three loci. Taken together, it can be inferred that lms1 might be a new mutation gene involved in lesion mimics. We are enlarging the mapping population and developing new markers for fine-mapping and cloning, and functional analysis of lms1 in the further.

ACKNOWLEDGEMENTS

This research was supported by the National Basic Research Program of China (Grant No. 2009CB119000), the National Science Foundation of China (Grant Nos. 31000094, 31100188 and 30970246).

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developmental age, and wounding on necrotic spot formation with \textit{Lsl1}. \textit{Dev Biol}, \textbf{93}: 381–388.


